ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL INSTITUTES OF HEALTH

1967 - 1968

NATIONAL INSTITUTE OF DENTAL RESEARCH

US NATIONAL INSTITUTE OF DENTAL RESEARCH

ANNUAL REPORT

July 1, 1967 - June 30, 1968

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

REPORT OF THE DIRECTOR

THE NATIONAL INSTITUTE OF DENTAL RESEARCH

July 1, 1967 - June 30, 1968

by

Seymour J. Kreshover

The dental research institutes program, for which grant support was first provided in FY 1967, has maintained steady progress. It has stimulated planning and development of broad, interdisciplinary, university-based centers of excellence in the sciences related to oral health. In the process, these institutes have attracted to the dental environment and to the solution of oral health problems the knowledge and skills of investigators in disciplines not heretofore intimately involved in dental research. Dental research institutes are in varying stages of development at the following universities: Alabama, Michigan, North Carolina, Pennsylvania, and Washington. Strong interest in establishing such interdisciplinary centers has been evidenced by four other universities or university consortia.

The report of the National Advisory Commission on Health Manpower included a number of observations and recommendations on dental education and research. In order to obtain a more adequate background on, and a better understanding of, the report, top Institute staff met with the two prominent dental educator-spokesmen who served as members of the Commission's Education and Supply Panel. The ensuing discussion focussed on the role of research and research training in dental education and developed possible approaches whereby NIDR might take a leadership role. It is anticipated that the reorganization of health activities within DHEW when fully implemented will permit NIDR to effectively provide leadership in this and other areas of dental education.

While the National Institute of Dental Research has had a long history of collaboration with the National Bureau of Standards, it has become increasingly evident that refinements in this interrelationship were mutually desirable. Accordingly, several meetings were held by top NIDR and NBS staff to explore possible new collaborative efforts and to define the role of each of the two participating institutions. It is expected that these meetings will result in a new agreement giving special emphasis to the research efforts at NBS supported by NIDR.

The organizational structure of the Institute continued to develop and evolve in a more meaningful fashion and to more adequately give visibility to its broad-ranging efforts and activities. Accordingly, the equivalent of branch status was authorized for the four extramural categorical program areas; i.e., Dental Caries and Hard Tissues Program, Periodontal Diseases and Soft Tissues Program, Oral-facial Growth and Development Program, and Materials Science and Special Clinical Studies Program. In order to emphasize the significance of applied studies, a new Biometry and Field Investigations Branch, reporting

directly to the Office of the Director and consisting of three sections (Biometry, Epidemiology, and Clinical Trials), was established. New sections in the intramural research area were set up as follows: a Virology Section in the Laboratory of Microbiology, and a Population Genetics Section and a Developmental Genetics Section in the Human Genetics Branch. The Institute became the first of the NIH complex to have its own personnel office, as a result of an experimental decentralization of the central NIH personnel management branch.

In cooperation with the NIH Management Policy Branch, an intensive study was made of work-flow, records, and other administrative procedures in the extramural programs area. This has resulted in a number of changes leading to greater efficiency and effectiveness.

The Institute and the dental scientific community were saddened by the death, in December, 1967, of Dr. Francis A. Arnold, Jr., former NIDR Director and a pioneer in fluoridation research on the prevention of dental caries. Institute personnel changes included the retirement of Dr. F. Earle Lyman from the position of Associate Director for Special Programs, the designation of Dr. Clair L. Gardner as the new Associate Director for Special Programs, and the transfer of Dr. Aaron Ganz from the Office of the Director, NIH, to serve as the NIDR Chief of the Office of Program Planning and Evaluation.

Dental Research Institutes

Designed to accelerate the development of scientific knowledge as an essential base for the advancement of dental research, dental education, and dental care, the program to establish dental research institutes in university settings is unique in drawing on total institutional resources. Its immediate aims are to facilitate the cooperation of biological, physical, and social scientists in the study of oral health problems of common interest and to have them interact with the educational programs of the parent universities by also functioning as members of academic departments. Additionally, the institutes will align themselves wherever practicable with other existing regional resources, including educational and research institutions, hospitals, computer facilities, Medlars regional centers, and primate centers.

Implemented one year ago, institutional grants have now been awarded to five universities: (1) the University of Alabama, (2) the University of Michigan, (3) the University of North Carolina, (4) the University of Pennsylvania, and (5) the University of Washington. Several other individual universities and groups of universities are currently developing proposals for the establishment of dental research institutes.

Since the Dental Research Institutes program represents, in the national interest, a unique concept of grant support involving rather large expenditures of public funds, specific guidelines for continuing evaluation by the granting agency and recipient institutions have been tailored to its specific requirements. This assessment is directed at assuring the orderly development of broadly based complexes bringing together a new mix of scientific and professional personnel with firm adherence to high standards of scientific merit and fiscal accountability.

Although sufficient time has not yet elapsed to expect substantive progress in meeting objectives, there have been indications that the participating dental schools are already benefiting from the rich cross-fertilization made possible by this new program.

Program Planning Office

This report encompasses the activities of the Program Planning Office, including those of the Reports and Analysis Section, during fiscal year 1968.

Analytical staff support was provided on a continuing basis to the Director, the Associate Director for Extramural Programs, the Associate Director for Special Programs, and the four extramural categorical program chiefs. In addition, a number of special projects were undertaken, as follows:

(1) An estimate of dental research manpower requirements for 1975 was prepared, based on the anticipated manpower needs of the following six categories of institutions that conduct dental research: dental schools, dental research institutes, the Federal establishment, other non-profit dental institutions, other non-profit, non-dental institutions, and private industry.

(2) A detailed analysis of the FY 1967 grants and activities of the Materials Science and Special Clinical Studies Program was prepared for presentation by the Area Chief to the Program Planning Committee and to the National Advisory Dental Research Council.

(3) A comprehensive analysis of how the 49 operating U.S. dental schools have utilized their General Research Support funds during the period 1962 through 1966 was completed. In addition to nine tables of basic data prepared for each of these institutions, summary tables and a commentary were also provided. This analysis was utilized to determine the impact of proposed GRS modifications on dental schools.

(4) Completed during the year was a major project analyzing the total NIDR extramural project awards made from FY 1967 funds for research and training purposes. This report provides the most comprehensive listing and analysis of these programs to date and will serve as a prototype and basis for regular annual publications in future years.

(5) Efforts continued to identify and obtain essential data on all current dental research activities being conducted in the United States and around the world. This kind of information is necessary to place NIDR's efforts in proper perspective by assessing the nature and magnitude of other related programs, thus providing a better basis for avoiding unnecessary duplication, improving coordination, and developing opportunities for various collaborative efforts. Broad coverage has already been obtained of such related activities of other NIH, PHS, and DHEW units. Cooperation has also been forthcoming from the Department of Defense, the Veterans Administration, and the Atomic Energy Commission. Major central resources such as the Scientific Information Exchange, the National Library of Medicine, and the Biomedical Research Information Service of the World Health Organization have been utilized in efforts to obtain a better picture of dental research

activities on a world-wide basis. In addition, special efforts have been devoted to a critical evaluation of the progress of the NIDR-supported Dental Research Information Center (DRIC), operated by the American Dental Association. This activity, established to provide a comprehensive source of information concerning the total U.S. involvement with dental research, is about to launch a major resurvey to obtain current data concerning the American dental research investigator community and its ongoing research activities. As a consequence of the importance of making this resurvey effort more directly responsive to NIDR's needs for reliable, overall information of this type, the Chief of the Program Planning Office has participated in a major way in redefining the objectives of DRIC and restructuring the original investigator and project questionnaires so that these key instruments can be more effectively utilized in obtaining the necessary information.

The Program Planning Office continued to serve in a liaison capacity between NIDR and related DRFR and DDH activities such as General Research Support, and Health Research and Health Educational Facilities construction programs. Contributions were made by this office to an intensive evaluation of the implications to NIDR of the April 1 reorganization of the health functions of DHEW.

Finally, a detailed evaluation was undertaken of the role and functions of the Program Planning Committee in order to achieve a more effective degree of utilization of Council members and other consultants in identifying and dealing with major problem areas that transcend the activities of the specific task-oriented committees of NIDR.

Information Office

The public's view of the dentist as an artisan-technician not only does an injustice to dentistry but also affects dental research. A major responsibility of the Information Office of the Dental Institute is to change this image. A changed attitude would not only invite more attention to oral disorders but would also create a greater public sensitivity to their importance and the need for preventive measures. By substituting a hope born of knowledge for current attitudes of indifference, apathy, and outright fatalism about tooth loss, we can greatly raise the level of oral health of the Nation.

Dental research is exceedingly diversified, current Institute-supported studies embracing a majority of the basic and clinical sciences. Reporting of research reflecting such a broad base of science helps to erode the common misconception of the narrowness of this field. Every opportunity is therefore vigorously sought to tell the story to the general public, the practicing dentist, dental educators, and the scientific community.

In vying for media attention, dental diseases patently are at a competitive disadvantage against such dramatic problems as heart disease and cancer; moreover, their relation to general health is not clearly perceived. Nonetheless, efforts this year have been successful in achieving greater coverage in the radio and television media as well as mass periodicals. One step in this direction has been to provide film clips on newsworthy research. For example, TV film clips and movies were prepared, in cooperation with

Merck Sharpe and Dohme, to publicize the encouraging experimental results with dextranase. This research report received wide publicity, including coverage on the Walter Cronkite Evening News Program. Also, news items appeared not only in the daily press, but in national publications such as This Week, Business Week, and Current Science.

For the first time this year, the Information Office took advantage of the opportunity offered by National Children's Dental Health Week, sponsored by the American Dental Association. This year's theme was "grow up smiling." NIDR provided the mass media with a feature story and photographs illustrating the role played by children in "research for the smile of health."

In another new service introduced this year, a feature article was prepared and mailed to approximately 3,000 company house organs throughout the country. Clippings now being received indicate good usage of this material.

National magazine queries, resulting in several articles about the Institute's research program, continue to be stimulated by the quarterly mailing of "NIDR Research Capsules," prepared for selected science writers. Time, Newsweek, Reader's Digest, Parade, Project Engineering, U. S. News and World Report, Consumer's Digest, U. S. Medicine, and Knight Newspaper Syndicate (which reaches 1.8 million readers) were among those reporting on dental research. Articles also appeared in the foreign press, including British, French, Italian, and Australian journals, and several Latin American publications. In the dental press, too, numerous articles appeared as a result of the monthly mailing of "research news from NIDR."

New opportunities to reach various segments of the public with information on research progress against oral disorders are also sought continuously. This year, for example, copies of the Institute's pamphlets on tooth decay and oral ulcerations were sent to several thousand teacher members of the American Association for Health, Physical Education, and Recreation. The pamphlets were also published in their entirety in TIC, a periodical found in the reception room of dental offices.

Related to the narrow image of dental research is the serious shortage of dental research personnel. A major effort has been undertaken to acquaint young science students, as well as science teachers and guidance counselors, with the variety of career opportunities in this field. The principal segment of this program is the production, under grant, of a motion picture and career brochure, illustrating the broad diversity of the dental sciences and focusing on the oral cavity as an integral system of the body.

Particular emphasis was also placed this year on the showing of a specially designed exhibit on the spectrum of careers in dental research. Although plans for showing the exhibit were modified due to budget restrictions, it was shown at four national and regional meetings of science students and teachers. A new brochure published this year, illustrating the wide scope of Institute grant-supported research, was made available at the exhibit. In addition to the large volume of material distributed from the exhibit, several thousand copies of the brochure, "The Expanding World of Dental Research" were sent to members of high school science clubs throughout the United States.

As part of the effort to present career opportunities, a member of the Information Office staff attended a Future Health Careers Day at St. Clair Shores, Michigan. Approximately 1,600 high school students attended to discuss health careers with representatives from many associations and agencies. In addition to the pertinent NIDR literature distributed at the meeting, arrangements were made to supply the Michigan State Health Council with literature and display material for a permanent exhibit panel for the Michigan State Health Careers Mobile Unit. This is a 50-foot trailer which travels throughout the state visiting senior high schools and junior colleges encouraging interest in the health careers.

A related service was introduced following a survey to identify appropriate honorary scientific societies to elicit membership interest in Institute programs. As part of this new continuing service, 7,260 pieces of literature, including leaflets on training and fellowship opportunities, as well as the two Institute brochures, were sent to the executive officers of 25 societies for distribution to their membership. Also, 700 pieces of training and fellowship literature were sent to 350 selected dental school faculty members, and former NIDR trainees and fellows.

The observance of the Institute's 20th Anniversary provided the Information Office an excellent opportunity to publicize progress of the past and plans for the future. In this connection. considerable encouragement and assistance was provided to outside authors for preparation of major articles for possible publication in early issues of the Journal of the American Dental Association, Science, Reader's Digest, and Today's Health. Other feature articles, with photos, were prepared by the Information Office for the NIH Feature News Service, NIH Photo Features, NIH Record, PHS World, Research for Health Column, News from NIDR and NIDR Capsules. A photo montage and selected individual photographs, together with a fact sheet and additional background material, were widely distributed to other audiences and science writers. This material was also sent to the deans of all dental schools and to the public relations officers of all NIDR grantee institutions with an accompanying letter requesting their cooperation in publicizing 20 years of progress against the oral disorders. This activity resulted in the mailing of 2,541 pieces of material to 1,596 addressees.

Responding to their expressed need for obtaining more information about NIH research programs, the NIDR Information Office mailed two of its principal brochures to each member of Congress. The publications, "The Expanding World of Dental Science" and "A Spectrum of Dental Research," excited wide interest and comment. Additional requests were received from members of Congress for an additional 2,420 copies of each publication for mailing to their constituents.

Due to budget restrictions, only one new scientific exhibit was developed by the Information Office this year. Designed in cooperation with the staffs of the Oral and Pharyngeal Development Section and the NIH Medical Arts and Photography Branch, the exhibit has created considerable interest. In competition with 80 other scientific exhibits, it was awarded second prize at the annual meeting of the American Dental Association. Although numerous requests for showing the exhibit were received, it was shown only at the

American Dental Association, American Association for the Advancement of Science, and the Federation of American Societies for Experimental Biology meetings. Altogether, the NIDR exhibited at nine meetings during FY 1968.

Another voluminous mailing of Institute literature was made this year to officers and research coordinators in universities, institutions, hospitals, and other organizations who might not be aware of the increasingly extended range of dental research. The 1,522 persons selected from certain mailing keys of the Division of Research Grants were sent 16,975 pieces of literature.

The Information Office recently revised four leaflets relating to the extramural programs. One was revised to inform dental students about the Institute-supported summer research opportunities in 1968. In this connection, an editorial was prepared for publication in the February issue of the Dental Student's Magazine, announcing these opportunities, and listing the schools providing the training. Sixty-thousand pieces of training and fellowship literature were mailed in February to the college addresses of over 10,000 dental school students.

In another effort to further acquaint the academic community with the scope of the extramural programs, the Information Office developed and made extensive distribution of the following informational items: Research and Research Training Supported by the NIDR; Directory of Resource Persons by Program Responsibility; Procedural Flow Charts for Research Grant Applications and Training Grant and Fellowship Applications; Statement of Function for the NIDR Extramural Programs, and Basic Science--The Core of Dental Research. Mailings of 2,055 of these items were made to 685 NIDR grantees, research coordinators and other selected university personnel. A total of approximately 161,000 pieces of literature was distributed this fiscal year, a 50 percent increase over last year.

The Institute's visitor program was especially active this year as a result of the "Open House" arranged in connection with the annual American Dental Association Meeting held in Washington, D.C. In addition, orientation programs and tours were arranged for approximately 357 visitors to the Institute. These programs are especially designed to meet the particular interest of each individual or group.

The Information Office also provided staff assistance in the Press Room at the American Dental Association Meeting and made necessary arrangements for four NIDR investigators to appear on the ADA closed circuit TV programs.

As the clearance center for manuscripts produced by Institute scientists, the Information Office processed 104 manuscripts and 53 abstracts during this year. The Office also prepared the NIDR component of the NIH annual bibliography, and the "Professional Staff of NIDR."

Internal reports prepared in whole or in part by the Information Office during FY 1968 numbered 116. Such documents include weekly reports to the Director of NIH of selected dental research advances and program developments, various annual reports, and revision of Departmental documents. Special material for budgetary and appropriation hearings prepared by the Information Office included

the Director's Opening Statement, narratives for the annual National Science Foundation report for Federal Funds for Science Series and the annual NSF Survey of Scientific Information, highlights of research progress, and other Congressional reports. Also, six speeches were prepared for key personnel of the Government.

In addition to the anniversary articles mentioned above, 39 articles were prepared for the NIH Record, PHS World, Research for Health, and Magazine Memo. Also, 22 press releases, summaries and announcements were produced.

Reaction to the Institute's first press conference, held in June, was enthusiastic, further attesting to the newsworthiness of present-day dental research. The Institute's Director and two members of the staff discussed new findings and new approaches to the prevention of dental caries. The Information Office provided press kits and TV film clips for media representatives in attendance.

Information plans for Fiscal Year 1969 will continue to emphasize recruitment. The motion picture now under production will be extensively promoted. Through widespread dissemination of research findings, using NIH information channels and direct media contacts, continued intensive efforts will be made to increase public understanding of the oral disorders and the hope held out through research.

Collaborative Research Office

The Collaborative Research Program continues its primary activity in support of research in the area of biomaterials. In addition, various intramural collaborative research activities are being supported by contract, and an initial step has been made toward extending the use of the contract, as a means of research support, to NIDR extramural programs.

Approximate expenditures for FY 1968 were: biomaterials research - $346,000; intramural collaborative studies - $160,000; interagency research agreements - $221,000; and extramural study in the oral-facial growth and development program - $8,777.

Biomaterials Research Program

This program is directed toward the development of new and more effective dental restoratives and other biomaterials for use in dentistry, including dental adhesive materials, characterization of the tooth and its function within the oral environment, and testing and evaluating new materials that may contribute to major advances in the practice of prosthetic and restorative dentistry. This effort is programmed by the multidisciplinary Biomaterials Research Advisory Committee; additional consultants are used as necessary.

The Committee does much of its work in programming at biannual meetings but, in addition, frequently reviews program elements through the year.

A systematic program to develop an adhesive dental filling material has been underway since 1964, and now shows increasing promise of success. Many

scientific disciplines are involved with the nature of adhesion mechanisms, of tooth surface and filling materials, the ultrastructure of enamel and dentin, the physical character of the cavity surface after drilling, improvement of existing dental materials and the fabrication of synthetic materials which relate to this program as well as evidencing useful application to other dental problem areas.

As a result of an Ad Hoc Workshop held in FY 1967, the investigation of barnacle cement was initiated to learn the composition and setting mechanism and to apply this knowledge to the development of an adhesive dental filling material. Experiments have shown that the barnacle cement adheres to all known materials except those containing copper. A substance which appears to be uncured cement has been extracted and chemical analysis of the cured cement has been initiated. To accelerate research interest in this material a pamphlet has been prepared describing the anatomy and physiology of barnacles, methods of growing barnacles in aquaria and means of harvesting the uncured cement. This pamphlet will be widely distributed to researchers and educators.

An Ad Hoc Workshop on Tooth Implants

An Ad Hoc Workshop was held at the NIDR to examine the method of replacing a tooth immediately after its extraction with a replica in plastic inserted immediately into the socket. The participants of the meeting represented the several disciplines which would be involved in a comprehensive targeted program to investigate this procedure which could be taken up by dentistry as part of its method of treatment. The group recommended that an aggressive program effort be initiated by the NIDR to develop that additional information which is needed to determine if this is a reliable clinical procedure.

Some additional comments were made concerning a wider application--such as those needing alveolar ridge reconstruction and support of periodontally involved teeth. It was agreed to consider only the single tooth implant at this time. It was suggested that a special advisory committee be used to guide this targeted program as this method would be effective in starting the program, monitoring it, and evaluating results.

ALPHA RESEARCH & DEVELOPMENT, INC. (PH43-64-502)

Title: "Investigations Leading to Improved Dental Restorative Materials"

Contractor's Project Director: Dr. Robert L. Patrick

Project Officer (NIDR): Dr. Robert J. Nelsen

Objectives: (1) To investigate the adhesion of rubbery polymers to hydroxyapatite, enamel, dentin and tough restorative materials, and to determine the degree of irreversible adsorption of a series of polymers to the various substrates; and (2) To investigate the permeability of restorative systems to water and diffusion of water through the rubbery interlayer.

Methods Employed and Major Findings: Four general systems were considered. The unlined model restoration; an acrylic rubber liner; a silane liner; and a dual-film acrylic rubber silane liner. Synergism was clearly shown in the case of the dual-film liner.

The stereoscanning electron microscope was utilized to evaluate film thicknesses in the model restorations. In addition, the surfaces of the model preparations were examined prior to application of liners and restorative resin.

Significance to NIDR Program: This investigation indicates that the use of a rubbery interliner acts as a sealant to the diffusion of fluids at the interface of the tooth filling and that the use of silane increases the effectiveness of this seal. There are also definite indications that the bond between the tooth and bulk restorative material is improved.

Proposed Course of Project: Scheduled for termination in FY 1969.

Current Annual Level: $28,131.

AMERICAN DENTAL ASSOCIATION (PH43-66-1127)

Title: "Preparation and Editing of a Dental Science Handbook"

Contractor's Project Director: Dr. Lon W. Morrey

Project Officer (NIDR): Dr. Robert J. Nelsen

Objectives: This dental science handbook is "designed for use by dental researchers, students and educators and will contain, in condensed form, the total spectrum of dental science and art including, but not limited to, the categorical areas of morphology, function, growth and development, treatment, materials, environmental factors of the mouth, pathology, public health and socio-economic statistics."

Each categorical area is being edited by a scientist of acknowledged leadership. He will select the text, illustrations, charts, etc.,for his area from the literature. The project director is Editor Emeritus of the Journal of the American Dental Association. It is anticipated that this will be published in 1969.

Proposed Course of Project: Scheduled for completion in calendar year 1968.

Date Contract Initiated: June 21, 1966.

Current Annual Level: $16,402 (estimate).

THE EPOXYLITE CORPORATION (PH43-64-548)

Title: "A Study of Epoxy Resin Dental Materials"

Contractor's Project Director: Dr. Henry Lee

Project Officer (NIDR): Dr. Herbert Swerdlow

Objectives: This contract is directed toward the study of epoxy resins for possible use as dental restorative materials.

Methods Employed: (1) Synthesis of specific resins customed for dental applications; (2) Measurements of physical properties of new resins, especially the reaction rate, water sorption, adhesive properties, color stability, hardness, and adhesion.

Major Findings: A number of filling materials which are free flowing, harden at tolerable temperatures, and have low curing shrinkage, are tough, hard and moisture resistant, have been developed. With these epoxy resins, the best adhesion to dentin is obtained with a citric acid - organo-functional silane - butanol pre-treatment of the tooth substrate. These materials are filled with glass beads, or aluminum, to increase their strength to approximate thermal expansion of the tooth. It has been found in this program that the bovine tooth is suitable as a test surface.

Significance to NIDR Program: The epoxy resin system appears to present one of the most lucrative means of developing a suitable adhesive dental restorative material.

Proposed Course of Project: This project will be continued at least until late FY 1969, and may be considered for an additional year.

Date Contract Initiated: April 28, 1964.

Current Annual Level: $53,500

HARRIS RESEARCH LABORATORIES (PH43-64-530)

Title: "An Investigation of the Mechanism of Adhesion to Teeth"

Contractor's Project Director: Dr. Anthony Schwartz

Project Officer (NIDR): Dr. Robert J. Nelsen

Objectives: To develop a dental restorative system that seals the tooth cavity completely and which provides a monolithic structure with respect to the strength of the restored teeth: Experiments directed toward the discovery of intermediately placed materials to bond acrylic to tooth substance have formed the central core of this project.

Major Findings: Polyurethane type compositions have been developed as adhesive liners for amalgam and self-curing acrylic restorations. Several polyglycol-diisocyanate compositions exhibited adhesion to human dentin when applied to moist teeth in vitro. Conventional acrylic or amalgam restorations became durably bound when applied on these liners within thirty minutes after they had been applied to the teeth. The composite restoration was water resistant and performed well in tensile, compressive and thermal shock adhesive tests.

The first experiments with the proposed standard test for adhesion were very promising. While some of the apparatus and a few manipulative procedures could be improved, a period of test procedure development should precede the introduction of formal changes.

Significance to NIDR Program: This contract is part of the total program directed toward an adhesive dental restoration. Specifically, it is mainly concerned with the use of conditioning agents and adhesive liners. Success in the development of an intermediate material which both the filling material and the tooth will bond is more likely to occur than will the development of a filling material that is itself directly adhesive to the tooth.

Proposed Course of Project: To be continued to FY 1969, and tentatively scheduled for expiration in April 1969.

Date Contract Initiated: April 29, 1964.

Current Annual Level: $32,417.

UNIVERSITY OF AKRON (PH43-67-1172)

Title: "A Study of Barnacle Cement"

Contractor's Project Director: Dr. Roger F. Keller

Project Officer (NIDR): Dr. Robert J. Nelsen

Objectives: (1) To obtain uncured barnacle cement for study aimed at assessing its value as a dental adhesive; (2) To analyze barnacle cement in terms of its chemical structure with a view to developing commercial methods of synthesizing the cement in large quantities. The synthesis may be of the total cement or of its adhesively functional subgroups.

Methods Employed and Major Findings: Work progressed in several distinct areas: (1) Anatomical, to further elucidate the cement flow path, isolate the responsible secreting gland, and prove that there are no glandular structures within the shell wall or base plate responsible for creation of cement; (2) Histological, aimed at the sectioning of the cement gland and identification of its functional parts; and also to examine the nature of the cement bond to human teeth; (3) Isolation and analysis of cured cement.

Future Work: It is anticipated that the uncured cement will be extracted and purified and the chemical structure will be determined through instrumental and other techniques. Studies will be made of the cured condition, the chemistry of curing and the rate of curing. In addition, the continuation of histological studies, both of the glandular secretions and of the barnacle tooth bonds is being made. This investigation will determine the feasibility of synthesizing barnacle cement which could prove to be a significant breakthrough in the field of adhesive dental materials, and, quite likely, may have application to the general area of tissue adhesives.

Significance to the NIDR Program: This investigation will determine the feasibility of artificially producing barnacle cement which could prove to be a significant breakthrough in the field of adhesive dental materials.

Proposed Course of Project: The project is scheduled to run through FY 1969 and will be considered for renewal on the basis of findings up until that time.

Current Annual Level: $34,640.

UNIVERSITY OF WASHINGTON

Title: "Histologic Comparison of Human and Swine Teeth for Operative Dentistry"

Contractor's Project Director: Dr. Gerald D. Stibbs

Project Officer (NIDR): Dr. Herbert Swerdlow

Objectives: The purpose of this project is to evaluate the feasibility of using Hanford Miniature Swine for future testing of dental materials and restorative procedures by operating on and making direct comparisons between human and swine teeth under identical or equivalent conditions.

Methods Employed: Recognizing the need to test new materials and procedures in other than human teeth, this program will compare the response of human teeth to swine teeth as they are exposed to conventional and experimental materials and procedures. Procedures for human subjects and the miniature swine will be the same. Human teeth to be removed for orthodontic reasons will be subject to the test procedure or material. The same operator will repeat the procedures on the miniature swine teeth. All subjects will be anesthetized during all operations. The response of the tissues of the human teeth will be compared to the response of the miniature swine and the observations will be correlated. After the interpretation and cross extrapolation of the results, it is expected that a model system will be developed for possible evaluation of new dental restorative materials utilizing miniature swine as a stand-in for man. Preliminary studies indicate the existence of a strong correlation between human and swine teeth in their response to dental treatment procedures.

Significance to NIDR Program: It is necessary to establish baseline information on the morphologic and histologic similarities and differences between human and swine teeth before swine can be used as experimental subjects for dental operations, although preliminary studies indicate the existence of strong correlation.

Proposed Course of Project: Scheduled to run through FY 1969. At the end of one year the project may be considered for renewal depending on results.

Date Contract Initiated: June 1968 (estimated).

Current Annual Level: $45,117 (estimated).

MONSANTO RESEARCH CORPORATION

Title: "Surface Coatings to Produce Permanent Silicate Cement Restorations"

Contractor's Project Director: E. A. McElhill

Project Officer (NIDR): Dr. Robert J. Nelsen

Objectives: There is good evidence that exposure of the surface of dental restorative materials to the mouth environment contributes heavily to their disintegration. This project is designed to determine the feasibility of protecting these surfaces from exposure to saliva and other fluids with a coating so that the integrity of the restoration may be maintained for a longer period of time.

Methods: Resins will be used as a coating material that have been developed as dental restorative materials. These resins have two of the required properties, namely, good mechanical toughness and good color stability. In addition, techniques are known for curing them in the environment of the mouth.

To obtain a strong bond of these materials to the surface of the silicate restoration, coupling agents developed in the aerospace industry for coupling polymers to glass surfaces will be used. These coupling agents are dual-functional and couple the resin directly to silicate surfaces.

The two major types of resinous dental restorative materials, namely, the methacrylate type and the epoxy type will be used as coating materials.

The restorations with coatings will be tested for solubility, discoloration, dehydration, compression resistance and tensile strength.

Significance to NIDR Program: If this project is successful it will allow an immediate and significant improvement of "on the shelf" materials.

Proposed Course of Project: This is a feasibility study to be started in late FY 1968 and be continued through FY 1969. It will not be continued longer than one year unless results warrant it.

Date Contract Initiated: June 1968 (estimate).

Current Annual Level: $21,000 (estimate).

UNIVERSITY OF TENNESSEE

Title: "Development of Standardized Toxicity Testing for Dental Materials and Products"

Contractor's Project Director: Dr. John Autian

Project Officer (NIDR): Dr. Herbert Swerdlow

Objectives: (1) To develop an "initial" or "preliminary" testing protocol for dental materials and products; (2) To develop a scoring system which may be helpful in delineating the degree of toxicity; (3) To perform toxicity tests on selected candidate materials and products which are being developed through contracts and grants from NIDR.

Methods Employed: Presently, the most perplexing problem in regard to the toxic potential of dental materials and products is the lack of a uniform manner in rating or scoring a toxic response. This program is designed to develop preliminary testing protocols for dental materials and products which will make use of a scoring system to delineate the degree of toxicity. When the scoring system has been defined, toxicity tests on selected candidate materials which are being developed in other programs of the NIDR will be performed as a means of screening.

Significance to NIDR Program: The experimental and testing procedures suggested in this proposal will, in effect, be the first systematic approach to the evaluation of acute toxicity of dental materials.

In the last several years, NIDR has encouraged and supported research in the development of new materials which may become useful as adhesives in one or more dental applications. Up to this date a number of candidate materials have evolved which are now being evaluated by physical, chemical and mechanical means. Results of research on implantable plastic teeth have also opened up a new avenue of possible dental treatment promising benefits to a large group of our population. These very recent developments add further support to the concept that the time has arrived for a systematic evaluation of dental materials and products.

Proposed Course of Project: Project is scheduled for implementation in late FY 1968 and will be considered for renewal dependent upon results.

Date Contract Initiated: June 1968 (estimated).

Current Annual Level: $24,810 (estimated).

MONSANTO RESEARCH CORPORATION (PH43-66-889)

Title: "Research on Composite Silicate Dental Cements"

Contractor's Project Director: Dr. Thomas Rockett

Project Officer (NIDR): Dr. Robert J. Nelsen

Objectives: Plastics and polymers offer promise of a new restorative material with improved properties, but there are still many unsolved problems associated with their use. This project is an attempt to improve existing dental materials by designing new compositions of silicate dental cements. To do this, cement-forming characteristics of a wide range of compositions are being studied.

Methods Employed: Frit composition is being varied systematically within a multicomponent oxide and fluoride phase equilibria system.

Major Findings: Several very promising compositions were discovered during the past work, and recently several more very interesting compositions were found. In addition to compositional work, the setting of commercially available materials and experimental cements are being studied by X-ray diffraction and electron microscope techniques in order to understand the setting mechanism of these cements. This information is being used to guide systematic variation of composition.

Last year several contributions were reported by this contractor. These included the identification of crystalline phases in the matrix of the silicate cements, development of a theory of setting mechanisms of cements and a ten-fold improvement of strength properties over some commercially available cements.

Accomplishment of the following tasks is planned for the coming year:

1. Complete screening of $CaO-Al_2O_3-P_2O_5-SiO_2$ frits.
2. Complete the refined tests on most of the superior experiments.
3. Supply to *in vivo* testing group at least two experimental cements.
4. Begin work on fluorine concentration
5. Begin work on additive materials.

Significance to NIDR Programs: This project is designed to help assure maximum benefit from current restorative systems until such time as better ones are developed and become universally available. In addition,

improved existing cements may well become part of the total dental restorative system of the future.

Proposed Course of Project: Scheduled for continuance through FY 1969, at which time the continuance will be determined based on results.

Date Contract Initiated: June 1, 1966.

Current Annual Level: $61,355 (estimate).

TYCO LABORATORIES

Title: "Non-Noble Metal Alloys for Dental Purposes"

Contractor's Project Director: Mr. Fritz Wald

Project Officer (NIDR): Dr. Robert J. Nelsen

Objectives: This project is directed toward determining the applicability of the copper-nickel-manganese system to dental purposes. The alloys to be investigated are used industrially as electrical resistance alloys; therefore, if they are suitable for dental use, they would be immediately available.

Methods: This program has been initiated to investigate the resistant alloys of copper, nickel and manganese. The physical property of this alloy system indicates that it may be quite possible to develop casting alloys which have the physical properties of the presently used gold alloys but without a high intrinsic cost.

Significance to NIDR Program: The successful development of an adhesive dental material will increase significantly the use of castable alloys in treatment procedures. Some of the newer techniques of prosthetic treatment now make use of cast posterior chewing surfaces. The presently available - gold and cobalt - chrome alloys are costly, difficult to cast or too hard and brittle, and thus have limited usefulness. In addition, this metal may become too costly for dental use.

Proposed Course of Project: This is a six-month feasibility study. Any consideration of renewal will be based on results.

Date Contract Initiated: June 1968 (estimate).

Current Annual Level: $24,460 (estimate).

THE REPORT OF THE DIRECTOR OF INTRAMURAL RESEARCH

THE NATIONAL INSTITUTE OF DENTAL RESEARCH

July 1, 1967 - June 30, 1968

by

Dr. Richard C. Greulich

Significant advances during the course of the past year have been achieved with respect to the two fundamental aspects of direct operating programs, namely program development and program execution. With respect to the latter, continuing and enthusiastic exploitation of individual and collaborative research efforts has provided additional evidence of the efficacy of the Institute's broadly based research approach, which includes a balanced spectrum of basic, clinical and applied studies. Highlights of the year's research progress are described below in terms of program execution, and are detailed, together with other items of progress, in the summary reports of NIDR's Laboratory and Branch Chiefs appended to this brief review.

Program Development

A variety of administrative changes have been implemented during the past year, calculated in the aggregate to provide a more conducive environment within NIDR for systematic pursuit and application of new knowledge relating to oral function and oral disease. Although several years' experience will be required to permit valid assessment of the efficiencies which these organizational changes are intended to foster, preliminary scrutiny of the research activities initiated during the past year inclines the writer to view them already as progressive steps. Hence, they will be described in this report.

Prominent among reorganizational changes made were those relating to the Institute's direct programs in the areas of epidemiology and biometry. In recognition of the essentiality of expanded capabilities for experimental design, automatic data processing and statistical analysis, and of the corollary essentiality of strengthening the Institute's expertise in the design and execution of field studies, a Biometry and Field Investigations Branch was established. This new research unit supplants the Institute's pre-existing Epidemiology and Biometry Branch, whose functions and personnel have largely been absorbed within it. Subsequently, three distinct sectional entities have been designated within the new Branch, namely the Biometry Section, the Clinical Trials Section and the Epidemiology Section. As its summary report will indicate, the activities of the Biometry and Field Investigations Branch have already been much expanded and refined as compared to immediately previous

years. A soundly-based field studies approach, backed by a high degree of competence in biometry, now permits systematic exploration of dental and oral problems in terms both of new modes of prevention and therapy, as well as of geographic pathology.

Other sections have been created within the framework of the Institute's laboratory research programs, notable amongst which are the Enzyme Chemistry Section, the Connective Tissue Section and the Cell Biology Section, all established during the year within the Laboratory of Biochemistry. Initiation of the Connective Tissue Section provides a functional visibility for the pre-existing excellence of individual and team efforts within the Laboratory in the area of mineralization, viewed as a normal - and all too frequently abnormal - characteristic of mesenchymally-derived tissue systems. Similarly, identification of an enzyme-oriented group serves to acknowledge pre-existing expertise in the area, and to emphasize the Institute's commitment to exploration of the fundamental nature of enzyme action. Systematically derived knowledge of this process is now generally conceded to be essential to gaining a full understanding of normal and abnormal biological function.

With respect to the Cell Biology Section, it may be noted that this group was previously attached to NIDR's Human Genetics Branch, as the Cellular Biology and Cytogenetics Section. Logical evolution of the research interests of this group, however, had been such as to turn its attention away from cellular inheritance, ultimately to focus on biochemical parameters of genetic expression in isolated cell populations. Conceptually as well as practically, therefore, it was deemed appropriate to merge this group's future activities with those of the Laboratory of Biochemistry.

Consequent to the loss of the Cytogenetics Section, careful scrutiny was also given to the research program of the Human Genetics Branch. Review of programmatic needs and individual talents within the Branch revealed the feasibility of designating two new sectional enterprises, namely the Population Genetics Section and the Developmental Genetics Section. These organizational titles accurately portray the clear recognition by the Branch of its continuing responsibility to expand its already rewarding program relating to genetic influences on human oral disease, expressed at the population level. Equally, there is implicit recognition of the need to explore to the fullest those aspects of genetics which can best be approached by analysis at the laboratory level, using animal models. In this context, promising emphasis is now being directed toward elucidation of genetic mechanisms governing organ differentiation in several mammalian systems.

The role of viral vectors in oral disease has long been a major area of research interest within the Laboratory of Microbiology. Demonstration by laboratory scientists during 1966 that a virus can combine with its antibody without losing infectivity provided even greater impetus to the study of virology, both with respect to the nature of the viral neutralization reaction, and to the role of viral infection in modulating immunological response. In concert with these research developments, and in recognition of the growing relevance of virology to our overall health mission, a Virology Section was established within the Laboratory of Microbiology during the past year.

Another significant administrative development pertained not to definition of a new organizational unit, but rather to reimplementation of an already existing one. Thus, personnel attrition having over the previous few years greatly reduced our capacity for research programs in X-ray crystallography, the Institute was faced with the need to assess the current and projected relevance of this technology to its mission. Accordingly, an intramural workshop was organized on the subject of "Crystallographic Concepts in Dental Research: Retrospect and Prospect." This informal, two-day symposium, the membership of which included experts in crystal structure drawn from Federal and academic sectors, as well as from NIH, concluded that many problems of great relevance to dental research remain to be explored, and strongly recommended that NIDR's intramural resources be geared to accommodate them. Recruitment of a new chief for the Crystal Chemistry Section in the Laboratory of Histology and Pathology was therefore undertaken, and, as the appended project reports will reveal, new and exciting dimensions of creativity are already evident.

In last year's summary report of intramural activities, the writer attempted to give visibility to a variety of individual research projects which, in the aggregate, focused on the oral environment. More specifically these dealt with selected aspects of endogenous and exogenous control of the oral apparatus. The writer employed this reportorial technique to emphasize his conviction that, to achieve better balance within the Institute's direct operating programs, establishment of an administrative unit concerned with oral physiology was both timely and appropriate. Accordingly, initial steps in this direction were taken during the year just past, in that a Physiology Section was organized, operating within the Office of the Director of Intramural Research. A significant increment in research creativity is well documented in the appended project reports from this Section, and augurs well for the future success of the unit. It is fully intended that, as budgetary circumstances will permit, this unit will ultimately evolve into a full-fledged Laboratory of Physiology.

Program Execution

Any effort to utilize this summary report as a vehicle for highlighting significant research advances is fraught with difficulty, if not danger. Thus, it seems prudent to preface any identification of specific research accomplishments with the caution that, whether the findings described below do indeed constitute significant steps forward in our quest for understanding of dental disease, will be determined only by the passage of time.

Without question, a greater effort than ever before has been devoted this past year to a concerted attack on the problem of dental caries. For prior research successes at NIDR and elsewhere, now clearly permit the development of rationally-based approaches to caries control. Particular emphasis has been concentrated on the natural history of a distinctive group of anaerobic streptococci which in animal model systems have been irrevocably implicated in smooth-surface dental caries. Evolving from previously accumulated evidence, exciting recent experiments have focused on a singular metabolic characteristic of these organisms, namely their production of an extracellular coating of a

polysaccharide derived from dietary sucrose. This coating, a long-chain dextran, appears to facilitate adherence of the organisms to the tooth surface as dental plaque. On the assumption that the adhesiveness of this gummy coating uniquely facilitates the apposition of organisms to the tooth surface, thereby leading to carious destruction, laboratory experiments have tested the effects of a dextran-hydrolyzing enzyme (dextranase) on plaque formation and caries induction. Incorporation of dextranase in both *in vivo* and *in vitro* systems has now been shown to prevent plaque accumulation and to inhibit the development of caries. These most promising laboratory findings suggest a potentially significant approach to the control of dental caries in man. Consequently, clinical field trials are to be undertaken very shortly.

Related studies of these plaque-forming streptococci have recently provided a deeper insight into their metabolic characteristics, particularly as they may specifically relate to the biochemistry of caries formation. Although requiring further experimental confirmation, results to date suggest that phosphate in the immediate environment enters these organisms very rapidly but does not leak out. Thus the cariogenic plaque effectively constitutes a metabolic sink for phosphate ion, much of which may well be derived from the subjacent enamel surface. Further evidence has been adduced which suggests that the intracellular phosphates are sequestered as negatively charged polyphosphates. Presence of the latter within the dental plaque may effectively convert it into a potent medium for cation exchange. It is tempting to speculate that this unique environmental situation contributes significantly to decalcification of the tooth in the caries process, and/or to mineralization of the plaque as it becomes converted to calculus.

Another type of microorganism, namely a filamentous diphtheroid (*Odontomyces viscosus*), has been implicated by past Institute studies in the etiology of periodontal disease. A potentially important advance in our capability to undertake systematic scrutiny of this disease entity has been achieved during the past year. Thus, after many unsuccessful attempts, cervico-radicular plaque formation and associated periodontal disturbances have been induced in hamsters by feeding them a diet containing starch, and by inoculating their mouths with diphtheroids of human origin.

Research on the natural economy of connective tissue continues to comprise a major endeavor within the Institute, in keeping with the clear-cut involvement of connective tissues in so many aspects of oral disease. New information of a basic character, relating to the mechanisms involved in fibrillogenesis of collagen and elastin, has recently been revealed. Prior study at NIDR had established that frank fibrillogenesis occurs as a consequence of extensive cross-linking of protofibrils, a mechanism known to involve conversion of lysyl residues in the polypeptide. An enzyme has now been identified in extracts of connective tissue that converts lysyl residues to allysine, presumably a first step in the cross-linking process. The activity of this enzyme is inhibited *in vitro* by treatment with a lathyrogen, at dose levels which previously have been shown to block collagen and elastin cross-linking *in vivo* and *in vitro*. Paradoxically, penicillamine, which also inhibits cross-linking, does not inhibit the enzyme, and so apparently exerts its deleterious effect at some stage in the process after allysine has been formed.

The effects of lathyrogens have also been explored with respect to their capacity for inducing abnormalities of growth and development in utero. These substances have now been shown to interfere with normal closure and fusion of the palatal shelves, so producing cleft palate. Thus, though presumably exerting their damaging effects through a mechanism related to their action on protein cross-linking, the lathyrogens join a long list of unrelated pharmacological agents which can induce palatal malformation.

Also within the area of connective tissue studies, research has been pursued and expanded with respect to identification and characterization of endogenous enzyme systems, which are assumed to play an important role in the formation, maintenance, and quite probably also, the destruction of various connective tissue components. In this context, emphasis has continued to be applied to characterization of collagenase which has been isolated from connective tissues under both normal and abnormal conditions. Of particular interest has been the recent demonstration that granulocytic leukocytes possess high levels of collagenase activity, probably associated with their complement of cytoplasmic granules. Evidence has now been developed which suggests that the collagenase is released to the external environment in response to a variety of stimuli, including tissue inflammation, and that only following release from the cell is the enzyme active in collagenolysis. In parallel studies, endogenous hyaluronidase has been identified in alveolar macrophages, and the environmental optima for its lytic action on hyaluronic acid have been defined.

Although an integral facet of NIDR's research mission, disorders of oral-facial development, including malocclusion, have long constituted a difficult area for systematic study. The prime hindrance to an orderly exploration of facial growth stems from the lack of reliable animal models or systems. A promising development in this regard has been the recent discovery that high doses of Vitamin D_2 administered to pregnant rabbits will regularly induce characteristic facial defects in the offspring. While this study is still in its initial phase, there is some reason to believe that the skeletal and oral defects will be sufficiently reproducible as to make this system highly useful for investigation of facial growth. Thus, detailed comparison of normal and abnormal rabbits may indeed provide leads to understanding of the growth mechanisms involved, and in turn these may ultimately be extrapolatable to the human situation.

Turning from research highlights, it is to be emphasized that, as in past years, all intramural professional staff have continued to acquit themselves with distinction in the generation and execution of research. Over 100 papers documenting their activities have appeared in the scientific literature during the past year. Moreover, in concert with their growing prominence in the scientific community at large, most of the professional staff have availed themselves of other avenues for communication, including presentation of newer research findings at national and international scientific meetings.

Reciprocally, as an ongoing aspect of NIDR's institutional responsibility for providing an environment conducive to creative scholarship, the weekly seminar program continued to provide a platform for the report of current research by scientists from NIDR, other NIH Institutes and Divisions, as well as from 59

other speakers, including 18 from foreign countries. As in past years also, this formally-structured program of seminars was complemented by weekly clinical conferences, and by a multitude of more program-oriented meetings at the Laboratory, Branch, or Section levels.

It is a matter of considerable pride to report that collaborative research activities have continued to play a major role in the execution of our research mission. Review of individual projects provides tacit evidence that the ramifications of dental research extend far beyond parochial and artificial boundaries. Thus, specific research projects were in progress or were completed during the past year involving active interplay between all of the Laboratories and Branches of NIDR, all of the categorical Institutes and research-related Divisions of NIH, several other research arms of the Federal government, and nearly two dozen institutions of higher learning in this country and abroad.

During the past year also the Institute has maintained its support of the activities of trainees and fellows including 7 Research Associates, 3 Clinical Associates, 3 Staff Fellows, 1 Postdoctoral Fellow and 1 graduate student in out-of-service training. On the international level, NIDR has also played host to 1 Visiting Scientist, 2 Visiting Associates and 1 Visiting Fellow whose individual research interests related to our activities in Microbiology, Histology and Pathology, Biochemistry, and Human Genetics.

A particular debt of gratitude is owed by the Dental Institute, and especially by the writer, to its Board of Scientific Counsellors. As in previous years, the Board's dedicated interest, enthusiastic support and wise counsel have provided further incentive for achievement, and have encouraged a profitable interchange of ideas relating to long-range program planning.

Report of the Physiology Section
National Institute of Dental Research
Summary Statement

The Physiology Section was established to fill a need within the Institute for investigations into systems of controlled and uncontrolled interaction between the organism and its environment, as related to oral biology. In this context are studies of neurophysiological mechanisms and pathways organized for processing of somatic sensation from the oro-facial region into the higher brain centers. This incoming sensory information is interacted with input from other modalities (e.g. visual, auditory, etc.) in association areas of the cerebral cortex and the response properties of nerve cells in such polysensory regions also is under investigation.

Another level on which organisms interact with each other as well as with their environment is during growth and development. The environment of a cell, particularly its biochemical environment, is an overriding determinant in deciding the type of development that it will undergo. A model for the environmental biology of development is found in the studies of the external control of the rate of differentiation and/or growth of the amoeboid sline mold, Dictyostelium discoideum.

The least organized bio-system under study in the Physiology Section is the response of caries-conducive streptococci to environmental parameters. A multiparametric approach is being adopted. Ongoing studies include: the dynamics of growth of these bacteria; the sequestration of phosphate (a main constituent of teeth) into the bacterial cell substance; the determinants of acquisition of capability to adhere to surfaces; and, related to this last, the dynamics of accumulation of cell-associated polysaccharides.

An area of investigation and service which interacts with all the foregoing areas of research is the application of computer science to the flow and control of laboratory information and experiments. This encompasses such diverse areas as on-line data reduction, investigations into better mathematical models for laboratory computations, and information retrieval.

Specific accomplishments in these several areas for the past year just passed are described in the following paragraphs.

Neurophysiological studies have been concerned with the functional properties of single cells in two central areas of the brain -- trigeminal brainstem nuclei and the cerebal cortex -- and how these properties relate to the response or reaction to painful sensations or experience. The data indicate that considerable modification of somatic input occurs at the first central relay nucleus in the trigeminal system. The masking of one sensation by another is related to the interaction between sensory stimuli and appears to involve the selection and filtering of input at many levels of the neuraxis. Modification of oro-facial pain involves such sensory interactions in the trigeminal system and the present studies offer physiological data that may explain these phenomena.

Other neurophysiological studies suggest that specific visual input is processed in middle suprasylvian gyrus, an association or polysensory area of cat cerebral cortex. The affective component of pain derived from the interaction of past painful experience and the perception of new pain is probably mediated in part by an alerting system projecting through medial thalamic structures and association areas of the cerebral cortex. This interaction requires that specific information about a painful stimulus, such as its shape, location in space, etc., be retained in polysensory areas of the central nervous system.

In investigating the biochemical factors initiating and controlling morphogenetic development of Dictyostelium discoideum, the main effort is concerned with those parameters affecting the rate of differentiation but not the quality. Based on the work to date it seems very likely that the ribose mononucleotides which accumulate in the cells only at the onset of differentiation provide a triggering message which starts the process. The mechanism by which this effect is mediated is currently under investigation. The demonstrated role of steroid metabolism in controlling the rate of morphogenesis also requires further clarification.

The multifaceted study of the environmental control of the cellular physiology of the caries-conducive streptococci has been quite fruitful. A coupling of phosphate accumulation to glucose fermentation and the control of this coupling by the environmental hydrogen ion concentration represents a new fundamental physiological phenomenon never described previously. Another unsuspected finding is the demonstration of massive inorganic polyphosphate accumulation by these bacteria. This last finding has important implications for any model to be constructed of the dynamics of caries production. The bacteria may represent an open-ended compartment for the sequestration of tooth phosphate.

Research on parameters of carbohydrate utilization in these streptococci are relevant to understanding the propagation of the carious lesion in two ways. The finding that glucose is predominantly metabolized to lactic acid by stationary plaque-forming streptococci establishes one of the critical chemical conditions which are part of the micro-environment of the tooth surface which contributes to the local dissolution of the tooth. The technological expertise gleaned in this study will be applied directly to the studies on growth and metabolism of these streptococci using the uniquely-labeled, high-specific activity sucrose we have synthesized. The sucrose will allow the study of the second parametric area of carbohydrate metabolism which is the polymerization of a portion of the sucrose into the adherent polysaccharide cell coating. It is apparently this dextran coating which allows the cells to adhere locally to the tooth surface, where the acid produced from the carbohydrate promotes tooth dissolution. The ability to select a population of adherent cells from normally non-cariogenic streptococci may indicate that the potential for adherence is possessed by a variety of oral microbiota.

Many of the above studies have been substantially aided and some even made possible by the application of computer technology and systems analysis to

the tasks of acquiring and analyzing the data. New methods have had to be devised as required. The major effort has been in the area of calculations of liquid scintillation counting data for radioactive tracer studies.

Serial No. NIDR-1 (66)
1. Office of the Director of Intramural Research
2. Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Functional Organization of the Trigeminal Brainstem Nuclei in the Cat.

Previous Serial Number: NIDR-82

Principal Investigator: Dr. R. Dubner

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 1 3/4
Professional: 1
Other: 3/4

Project Description:

Objectives:

Somatic sensation from the oro-facial region reaches higher brain centers in the thalamus and cerebral cortex via pathways distinct from somatosensory projections from all other parts of the body. These pathways comprise the central connections of the trigeminal system which are also unique in their close proximity to the reticular formation of the brainstem. The present studies were designed to investigate the functional properties of cell groups within trigeminal brainstem nuclei and to study the modifying influences of central and peripheral inputs on these cells.

Methods Employed:

Cellular activity in trigeminal brainstem nuclei was studied utilizing extra-cellular microelectrode recording techniques in cats anesthetized with chloralose or nembutal. Peripheral receptive fields were located with natural stimuli such as a fine camel hair brush, were then stimulated electrically, and the cellular responses interacted with peripheral input (flash or click) and central input (cerebral cortex) and thalamic stimulation. Precise

electrode localization was determined histologically with the aid of a dye-marking technique. The excitability of primary afferent trigeminal fibers was tested by placing a stainless steel microelectrode at the junction of the trigeminal nerve and the main sensory nucleus in the brainstem. The antidromic compound action potential evoked by stimulation of this site was recorded from the cut ends of the infraorbital or mental nerve. Conditioning visual, auditory, somatic or central stimuli were employed to determine the presence of primary afferent depolarization evoked by such stimuli.

Major Findings:

Excitability changes in the terminals of trigeminal afferent fibers were produced by cortical stimulation and by electrical stimulation of the face or trigeminal peripheral nerve strands. Light flash stimuli produced small and variable excitability changes in these terminals. However, stimulation of the optic disc resulted in consistent excitability changes with a time course similar to the other conditioning stimuli. Stimulation of the dura mater or pia vessels on the cortical surface also produced primary afferent depolarization. Primary afferent depolarization still was evoked by stimulation of the optic disc, or the somatosensory areas of the cerebral cortex, after contralateral trigeminal nerve section proximal to the Gasserian ganglion.

Significance to Dental Research:

The data indicate that considerable modification of somatosensory input occurs at this first central relay nucleus in the trigeminal system. The masking of one sensation by another is related to the interaction between sensory stimuli and appears to involve the selection and filtering of input at many levels of the neuraxis, including the first synapse. Modification of oro-facial pain (e.g., audio analgesia, the rubbing of a painful area, etc.) involves such sensory interactions in the trigeminal system; the present studies offer physiological data that may explain these phenomena.

Proposed Course of Study:

Further research will include studies on pathways of visual and auditory input to trigeminal brainstem nuclei. In addition, patterns of activity initiated by large and small fiber components will be studied utilizing computer methodology and the responses to these stimuli will be interacted with nonsomatosensory input.

1. Dubner, R.: Interaction of Peripheral and Central Input in the Main Sensory Trigeminal Nucleus of the Cat. Exptl. Neurol. 17: 186-202, 1967.

Serial No. NIDR-2 (64)
1. Office of the Director of Intramural Research
2. Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Interaction of Sensory Stimuli in Association Areas of Cerebral Cortex in the Cat

Previous Serial Number: NIDR-83

Principal Investigator: Dr. R. Dubner

Other Investigators: Dr. B. Dow and Mr. F. J. Brown

Cooperating Units: None

Man Years:

Total:	2 1/2
Professional:	2
Other:	1/2

Project Description:

Objectives:

The association areas or "non-primary" projection areas of the cat cerebral cortex are responsive to stimuli of polysensory origin (e.g. visual, auditory, somatosensory, etc.), and exhibit response characteristics different from those of primary sensory areas of the cerebral cortex. Our previous studies indicate that cells in these association areas, are unequally responsive to different stimuli, and that cellular mechanisms are available for the interaction and processing of sensory-specific information. Other investigators, however, have suggested that these association areas are merely concerned with the "arousal" and "orientation" of the animal and do not receive modality-specific input. The present studies were designed to investigate further the response characteristics of neurons in one association area (middle suprasylvian gyrus) to discrete visual stimuli.

Methods Employed:

Cellular activity in the middle suprasylvian gyrus of the cerebral cortex was studied with microelectrodes in cats anesthetized with

chloralose. Receptive fields and response characteristics of these cells were ascertained using moving and stationary light sources projected on a tangent screen. Stimuli included circular spots (1-8° in diameter) and variously oriented rectangular bars (1/4-2° width, 2-6° length), as well as straight edges of variable length (up to 20 degrees).

Major Findings:

Nearly three fourths of the cells encountered in middle suprasylvian gyrus show specific responses to visual stimuli. Three distinct response patterns are apparent: 1) Edge detector cells give brisk responses to specifically oriented edges moved in preferred directions across sharply delimited positions in their receptive fields, and respond poorly to stationary stimuli. 2) Spot detection (? light detector) cells fail to respond to moving stimuli, but give strong off responses (and sometimes weak on responses as well) to properly positioned large spots. Field sizes for these cells range from as small as 3-5 degrees up to 20 degrees or more. Smaller spots produce weaker (but similar) responses over the same receptive field. Binocular facilitation is common, with off responses from one eye combining with on/off responses from the other eye to give a strongly off field with scattered weaker on responses overlapping in the center. 3) Complex center cells give nonspecific responses to moving edges, most typically to edges of any orientation moving towards the receptive field center (i.e. centripetally). Stationary stimuli produce strong on/off responses in the receptive field center (5-20 degrees) and somewhat weaker off responses over a more extensive surrounding area (greater than 40 degrees in some cases). Optimal stimuli have yet to be determined for this latter group of cells, but variously oriented bars tend to be more effective than spots.

Significance to Dental Research:

These data suggest that sensory specific visual input reaches middle suprasylvian gyrus, a "non-primary" or association area of cat cerebral cortex. The results are at a variance with the hypothesis that all the association areas of cat cerebral cortex receive identical inputs and are devoid of sensory specificity. From our previous results, it is known that neurons in these areas respond differentially to multiple sources of input and exhibit cellular synaptic patterns which may be important in sensory integration.

The response or reaction to pain appears to involve many levels and many parts of the central and peripheral nervous systems. It has been suggested that the affective component of pain derived from the interaction of past painful experience and the perception of new pain is mediated in part by an alerting system projecting through medial thalamic structures and association areas of the

cerebral cortex. This interaction requires that specific information about a "painful stimulus" such as its shape, location in space, etc., be retained in the central nervous system. The present studies indicate that one association area of the cat cerebral cortex, the middle suprasylvian gyrus, receives specific visual input (and possibly specific auditory and somatosensory input) as well as non-specific input form medial thalamic structures. This polysensory convergence from multiple central and peripheral sources provides the association cortex with integrative capabilities necessary for participation in sensory responses such as pain, taste, and olfaction, where affective phenomena play a prominent role.

Proposed Course of Study:

Further clarification of the response properties of these cells is planned. Efforts will be made to establish the distribution and interaction of cells in this cortical region, as well as to determine the nature of the input from thalamus, primary visual cortex, and corpus callosum.

Computer methods will be employed to analyze time distributed cellular events occurring under conditions of unaltered and altered input to association cortex. Plans include "on-line" operation using a computer-controlled light source, with new stimulus parameters being determined on the basis of feed-back from previous responses.

Part B. Publications

1. Dubner, R. and Brown, F. J.: Response of cells to restricted visual stimuli in an association area of cat cerebral cortex. Exptl. Neurol. 20:70-86, 1968.

Serial No. NIDR-3 (60)
1. Office of the Director of Intramural Research
2. Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: The Biochemistry of the Differentiating Cellular Slime Mold, Dictyostelium discoideum

Previous Serial Number: NIDR-6

Principal Investigator: Dr. M. I. Krichevsky

Other Investigators: Dr. B. M. Chassy

Cooperating Units: None

Man Years:

Total:	2 1/2
Professional:	1 1/4
Other:	1 1/4

Project Description:

Objectives:

To investigate the biochemical factors initiating and controlling morphogenesis of Dictyostelium discoideum. Presently the main effort is concerned with the factors affecting the rate of differentiation but not the quality. Special emphasis is being placed on the earliest biochemical changes observable with the onset of the morphogenetic process, since the nutritional factors increasing the rate of differentiation can be shown to do so during the initial period under study.

Methods Employed:

All are standard techniques routine to the types of studies herein described.

Major Findings:

Ribose mononucleotides accumulate in the cellular slime mold at the onset of differentiation (and only then). These materials are stimulatory to the rate of differentiation as are the 5'-esters when supplied exogenously. The specificity of the mononucleotide stimulation of the rate of differentiation in the cellular slime mold was found to be complete for the whole molecule. That is, the stimulant must consist of purine or pyrimidine base, pentose moiety, and a phosphate group. Changing the nature of the purine or pyrimidine or substituting deoxyribose for ribose or moving the phosphate from the 5'-position to 3' affected the stimulation only quantitatively. Leaving off one or more parts of the tripartite molecule resulted in either loss of stimulation or actual inhibition of the rate of differentiation.

A compound which is known to inhibit steroid biosynthesis in mammals was found to inhibit the rate of differentiation in D. discoideum. The inhibition could slow the completion of differentiation from 1.5 days to as much as 7 days. Analysis of cells entering the differentiation stage by gas chromatography indicated that they already contained high levels of steroid. Therefore, the same assay was used to investigate the ability to arrest growth and steroid biosynthesis. It was found that growth was inhibited less stringently than is steroid biosynthesis. The steroid is formed after the majority of growth takes place.

Significance to Dental Research:

The phenomena of nongenetic functional changes in cell types such as embryological differentiation, microbial spore formation, induced enzyme formation, cancerous de-differentiation, aging, etc., are an integral and fundamental part of the life history of all organisms. It is desirable to have information regarding the biochemical factors influencing the initiation and course of such changes, since many aspects of medical phenomena are intimately affected by these functional differences among cells and tissues. An example of anomalous differentiation of concern in dental research is the cleft palate syndrome.

Proposed Course of Study:

Experiments will be performed to determine whether the 2'-, 3'-mononucleotides are converted to the 5'-mononucleotides during differentiation. Adenosine-5'-monophosphate metabolism will be studied to gain insight as to the mechanism of action of the 5'-esters.

1. Krichevsky, M. I.: Some uses of computers in chromatographic technology, present and future. In Automation in Analytical Chemistry. New York, N. Y., Mediad Inc., 1967, 453-455pp.

2. Krichevsky, M. I. and Love, L. L.: Accumulation of mononucleotides in washed suspensions of myxamoebae of Dictyostelium discoideum. J. Gen. Microbiol. 50:15-21 (1968).

3. Krichevsky, M. I., Zaveler, S. A., and Bulkeley, J.: Computer-aided single or dual isotope channels ratio quench correction in liquid scintillation counting. Anal. Biochem. 22:442-464, 1968.

4. Krichevsky, M. I. and Keyes, P. H.: Considerations in designing a system for developing models. Currents in Modern Biology. In press.

Serial No. NIDR-4 (66)
1. Office of the Director of Intramural Research
2. Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Energy Dependent Phosphate Accumulation by Streptococci Implicated in Smooth Surface Caries

Previous Serial No.: NIDR-29

Principal Investigator: Dr. J. M. Tanzer

Other Investigators: Dr. M. I. Krichevsky

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

The overall goal of this project is an understanding of the regulation of the interplay of PO_4 metabolism and the formation of acid and polysaccharide from sugar by plaque-forming streptococci implicated in smooth surface caries.

The immediate goal of the present phase of study is to establish whether these plaque-forming streptococci are capable of forming polyphosphates. The conditions which regulate the formation of polyphosphates by these cells will be delineated in respect to the critical variables of *in vivo* plaque.

Methods Employed:

Streptococcal cultures were maintained, grown, harvested, and experimentally incubated as stationary phase cells. Cells were exposed to o-$^{32}PO_4$, challenged with glucose, and the accumulation of P^{32} monitored. These techniques were previously detailed (Tanzer, Annual Report 1967).

Two basic experimental stratagems were employed:

1. In order to determine whether phosphate accumulation was directly coupled to the accumulation of exogenous carbon-containing fragments, cells were challenged with glucose-U-^{14}C and the simultaneous uptake of ^{32}P and ^{14}C was monitored.

2. In order to determine whether phosphate was accumulated into a pre-existing carbon-containing cellular pool, cellular carbon-containing pools were prelabeled by growing the cells in glucose-U-^{14}C. Then ^{32}P uptake was monitored when cells were challenged with carrier glucose.

   After the period of phosphate uptake in response to challenge by exogenous glucose, cells were either sequentially extracted with cold and hot $HClO_4$ or they were extracted with NaOH.

   Cell extracts were analyzed by column chromatography on Sephadex G-25 and on Dowex-1-formate. Fractions eluted from the columns were counted for ^{32}P and ^{14}C radioactivity. ^{32}P counts were resolved from ^{14}C counts by a computerized quench correction technique.

   A thin layer chromatographic technique was developed which permitted separation of polyphosphates of different size. Techniques were developed which permitted elution of ^{32}P containing fragments independent of ^{14}C containing fragments.

Major Observations:

1. About 50% of the phosphate accumulated by the organism under study is not extractable in cold $HClO_4$.

2. Most ^{32}P incorporation into cells appears not to require simultaneous incorporation of ^{14}C into the same cellular pools.

3. A large fraction of the ^{32}P incorporated into cells passes into a pool which contains no pre-existing ^{14}C. Observations 2. and 3. are consistent with the hypothesis that this caries-active streptococcus is capable of synthesizing a large quantity of polyphosphate -- a polymer of phosphoryl residues, free of carbon.

4. By thin layer chromatography approximately 20% of the total ^{32}P incorporated by the cells behaves as if it were polyphosphate of 16 phosphoryl residues or greater in dimension. This permits the tentative conclusion that 20% of the ^{32}P accumulated exists in phosphate polymers of at least 1500 molecular weight units.

5. No polyphosphate appears to be excluded from the void volume of Sephadex G-25. Hence, no polyphosphates are apparent with a greater molecular size than about 5000 molecular weight units.

Conclusions 2. through 5. must be viewed as tentative and subject to confirmation by subsequent experimentation.

Significance to Dental Research:

Streptococci of the variety under study have been shown to form plaque on the smooth surfaces of teeth. Smooth surface carious lesions occur only under such bacterial growths. What are the aspects of metabolic behavior of the plaque-forming microorganisms which allow their establishment, survival and growth on the tooth surface, and their invasion of the tooth substance? Does the tooth modify the metabolic activity of these etiologic agents? These issues have come into focus. If answers can be achieved in respect to the demands, capacities and regulation of metabolic activity of these cells, one might be able to more fully comprehend plaque-associated disease and design more meaningful interceptive measures. This work is addressed to these ultimate goals.

Some specific questions and points of significance can now be crystallized. From previous data (Tanzer, Annual Report 1967) it was seen that these plaque-forming streptococci accumulate phosphate at very high rates. We now know that the flux of phosphate across the cell membranes of these cells is unidirectional. Hence, phosphate in the environment of these cells not only moves into the cells at very high rates but it does not leak out. These cells, living on the surface of enamel, therefore, constitute a sink, continuously renewed, for the movement of phosphate.

Thus, a critical question arises in view of the avidity of these cells for phosphate and the unidirectionality of phosphate movement: Must these cells draw upon the phosphate pool of the tooth in order to meet their demand for phosphate?

The tentative establishment of the synthesis of polyphosphate by these cells directs attention to at least two points.

1. Cellular synthesis of polyphosphates is classically an event which occurs only in non-growing (stationary) cell populations. The synthesis of polyphosphate allows cells to accumulate phosphate throughout their life cycle.

2. The presence of the highly negatively charged polyphosphate within the plaque cell may effectively convert it into a potent cation exchange resin. This effect could be important in either of the processes of decalcification of the tooth in caries or in mineralization of the plaque in calculus formation.

Proposed Course of Study:

The project to date has employed a washed non-growing cell system. This work will be continued in this model and extended in a direction such that the behavior of the intact plaque resident on the tooth surface will be approximated. Preliminary studies have investigated the feasibility of proceeding in this direction.

Preliminary experiments:

1. Growth of intact plaque. Quantitative measurements of *in vitro* plaque growth on a smooth inert surface have been shown feasible. Under the conditions employed growth of plaque is linear rather than exponential and is at a rate of 5 mg wet weight/day/cm^2 surface area. Since the dry weight of this plaque and its total DNA content increase in parallel with the wet weight, there appears to be no change in the plaque composition with time. The linear rather than exponential growth of this system attests to the diffusion limitation of behavior of intact plaque.

2. Fowler has produced easily manipulated disks of hydroxyapatite which can serve as conveniently symmetrical "teeth" of known composition. Plaque growth on such disks is rapid and produces demineralization of the disk. We therefore seem to be in a position in which we can characterize the differences, if any, of plaque growth on an inert surface in contrast to plaque growth on a "tooth". We are also in a position in which we can define the dissolution rate of "tooth" under plaque and the associated rates of acid production and PO_4 accumulation by the plaque.

Part B not included

Serial No. NIDR-5 (67)
1. Office of the Director of Intramural Research
2. Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: The Metabolic Fate of Glucose Catabolized by Stationary Phase Streptococci Implicated in Smooth Surface Caries

Previous Serial No.: None

Principal Investigator: Dr. J. M. Tanzer

Other Investigators: Dr. M. I. Krichevsky and Dr. P. H. Keyes

Cooperating Units: None

Man Years:

Total:	1/4
Professional:	1/4
Other:	0

Project Description:

Objectives:

It seems unlikely that frequently ingested sugar solutions would support a significant rate of plaque growth. It is also unlikely that oral fluids would supply nutrients required for rapid plaque growth. Hence, it is probable that plaque in vivo is growing at very slow rates. Some indirect experimental data support this assumption.

Strangely, almost no data is available concerning the metabolic fate of catabolized sugar by plaque-forming streptococci. The information which is available, however, describes rapidly growing cells. In view of the purported role of acid products of bacterial metabolism in the etiology of caries, the goal of this project was a definition of the acid products of sugar fermentation by stationary phase plaque-forming streptococci.

Methods Employed:

Streptococcal cultures were maintained, grown, harvested and experimentally incubated with glucose-^{14}C as stationary phase cells. These techniques were previously described (Tanzer, Annual Report, 1967). The products of fermentation were separated by Dowex-1-formate anion exchange chromatography and by partition chromatography upon Celite-535 columns. Fractions eluted were assessed for ^{14}C radioactivity.

Major Findings:

1. Lactic acid comprises about 90% of the fermentation products of glucose.

2. Acetic acid and CO_2 are virtually absent as fermentation products.

3. About 6% of the metabolic products of fermentation are represented by a carboxylic acid(s) of 3C or greater chain length.

4. No appreciable ^{14}C becomes incorporated into cell substance other than as mono and diphosphate esters of the Embden-Myerhof path or intracellular amylopectin-type polysaccharide. This datum supports the conclusion, reported independently (NIDR-4) in this document, that these cells form polyphosphates.

Significance to Dental Research:

This work establishes that the acid produced by stationary plaque-forming streptococci is predominantly lactic acid. It thereby establishes one of the critical chemical conditions which exists on the surface of the tooth. It is a condition which is germane to the definition of the environment contributing to dissolution of the tooth.

One bonus results from showing that 90% of the products of sugar fermentation by plaque-forming streptococci is lactic acid. Since it is well established that 2 net moles of ATP are produced in the conversion of 1 mole of glucose to 2 moles of lactic acid, at least 1.8 moles of ATP are produced per mole of glucose catabolized by these cells. If one knows the rate of acid production by such cells, that rate is numerically equal to the number of net ATP moles produced in fermentation. Thus, by measuring the acid production rate, one can evaluate the energetic concomitant of any energy consuming process under investigation.

Proposed Course of Study:

It is felt that the goal of this project has been achieved. Thus, there are no present plans for continuation of this study.

Part B not included.

Serial No. NIDR-6 (67)
1. Office of the Director
   of Intramural Research
2. Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Growth and Metabolism of Cariogenic Streptococci

Previous Serial No.: None

Principal Investigator: Dr. Bruce M. Chassy

Other Investigators: Dr. M. I. Krichevsky and Dr. J. M. Tanzer

Cooperating Units: None

Man Years:

Total:	1 1/4
Professional:	1
Other:	1/4

Project Description:

Objectives:

The overall goal of this project is to provide an understanding of the relationship of diet, growth, and culture conditions in the formation of plaque and caries by human oral cavity streptococci.

Specific goals of this project are 1) to synthesize (glucose-^{14}C) sucrose and (fructose-^{14}C) sucrose 2) determine the fate of sucrose (using the isotopically labeled sucroses synthesized above) in cariogenic streptococci under a variety of growth conditions (i.e. under varying conditions of pH, concentration of substrate, temperature, time of incubation, etc.) and 3) to evaluate the effect of exogenously supplied dextranase on sucrose utilization.

Methods Employed:

Sucrose (isotopically labeled in either the glucose or fructose residue) is synthesized from uniformly labeled sucrose by a combination of chemical and enzymatic methods. These specifically labeled sucroses have heretofore not been available at high purity and specific activity.

Bacterial growth experiments, on a pure strain of cariogenic streptococci, have been performed in a temperature controlled pH-stat. Uptake of $^{32}PO_4$, production of DNA, acid, and polysaccharide has been followed by chemical and instrumental methods.

Major Findings:

1. Synthesis of labeled sucrose has been improved over older methods.

2. It is possible to use controlled growth instrumentation as a tool in investigating the growth of cariogenic streptococci.

3. A dextran-producing cariogenic streptococcus has been found to grow linearly rather than exponentially on sucrose. This organism grows in the expected exponential manner on other carbohydrates including mixtures of glucose and fructose.

Significance to Dental Research:

An approach to effective control of the causes of smooth surface caries necessitates a basic understanding of metabolism, growth and plaque formation by caries specific bacteria. An examination of the unique role of sucrose in its effect upon the growth characteristics, dextran (polysaccharide) formation, and plaque formation should suggest fundamental methodology for oral hygiene as well as contribute to our understanding of the complex interaction of various components present in the oral cavity.

Proposed course of Study:

1. To delineate the sucrose metabolism of a cariogenic streptococci by existing methodology.

2. Study the effect of various factors on growth and development of cariogenic streptococci (i.e., dextranase, pH, inhibitors).

3. Develop an automated, on line, computer controlled evaluation system that will monitor and control various experimental parameters. Such a system, applicable to bacterial growth and enzymology, will allow rapid and precise experimentation on some of the factors mentioned above. The system will be used to develop a mathematical model to help understand linear growth as well as growth and acid production under the conditions found in the mouth. The introduction of modern data gathering and reduction techniques will not only greatly facilitate this study but should be of general interest to the biomedical research community.

Part B not included

Serial No. NIDR-7 (67)
1. Office of the Director of Intramural Research
2. Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Selection of Plaque-Forming Mutants of Streptococci.

Previous Serial Number: None

Principal Investigator: Dr. J. M. Tanzer

Other Investigators: Mr. R. M. McCabe

Cooperating Units: None

Man Years:

Total:	1/4
Professional:	1/4
Other:	0

Project Description:

Objectives:

How do people become infected by caries-active plaque-forming streptococci? Infection could conceivably occur from the external environment of the host. It could also occur via mutation of caries-inactive streptococci to caries-active variants. This study's goal was to attempt to select plaque-forming mutants from a previously non-plaque-forming population of cells.

Methods Employed:

A number of strains of plaque-forming and non-plaque-forming streptococci were grown in broth containing sucrose. A steel wire was introduced to the broth and the wire was transferred daily to a tube of uninoculated culture broth. Plaque growth was assessed by a semi-quantitative visual method at the end of 3 series of 5-day wire transfers. After 3 series of transfers, the microorganisms were cultured in broth free of sucrose.

On return to broth containing sucrose one could test whether experimentally observed changes were heritable or merely a result of enzymatic inductions in the presence of sucrose.

Major Observations:

1. With non-plaque-forming bacterial strains 2M2, 2M2R, and 903-1600, the passage of a bacteria-covered wire from tube to tube in sucrose-containing broth selects the most adhesive fraction of a population of cells.

2. This results in a population with a greater ability to colonize on a smooth surface, i.e., to form plaque.

3. Two conditions appear important for the selection process: the presence of sucrose and a means of preferential transfer of more adhesive cells.

Significance to Dental Research:

These experiments suggest a possible means whereby plaque-forming strains evolve from non-plaque-forming inhabitants of oral cavity, i.e., by natural selection for growth on the tooth's smooth surfaces of more adhesive microorganisms under the influence of sucrose.

Proposed Course of Study:

If one were to implant a tagged non-plaque-forming strain in an experimental animal and show that one could select mutants which were sufficiently adhesive to form plaque and cause caries -- then the origin of caries-active microorganisms from previously caries-inactive ones would have been demonstrated.

Part B. Publications

Tanzer, J. M. and McCabe, R. M. Selection of Plaque-Forming Streptococci by the Serial Passage of Wires Through Sucrose Containing Broth. Arch. Oral Biol. 13:139-143, 1968.

Serial No. NIDR-8
1. Office of the Director of Intramural Research
2. Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Laboratory Information System

Previous Serial Number: None

Principal Investigator: Mr. John J. Wilson

Other Investigators: None

Cooperating Units: Division of Computer Research and Technology

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

1. To design and develop a computer system capable of on line instrument monitoring and data processing.

2. To investigate the mathematical properties of laboratory experiments and develop computer solutions.

3. To design an automated information retrieval system to allow for the formation and utilization of the data bank on microbial taxonomy now being developed.

Methods Employed:

The techniques of systems analysis and computer programming normal to these needs.

Major Accomplishments:

1. Computer Time Sharing.
A study was conducted of the available computer time sharing services. The features and costs of each system were evaluated

with respect to the requirements of the Institute. Recommendations were made to the Scientific Director for initiation of two of the services. These services should be in operation at NIDR in May, 1968.

2. Scintillation Counting.
A series of programs were designed and written to provide computer processing of the data from two Packard Tri-Carb scintillation counters which were acquired last year.

The programs were written to determine the disintegration per minute of one or two isotopes counted in a scintillation cocktail. The programs were written in Fortran IV and are being run on the IBM 360/50 computer system, Division of Computer Research and Technology, NIH.

3. Phosphate Uptake.
A program was written in Fortran IV to determine the phosphate uptake and acid production rate for hamster-type plaque forming caries-active streptococcus.

4. Information Retrieval.
A computerized system was designed to provide personnel budget allocation by project number. The program was written by DCRT personnel according to design specifications.

The background obtained in this effort is directly applicable to the microbial taxonomy problem.

5. On Line Computer.
Process control computer characteristics were investigated. The laboratory experiments and instruments to be interfaced with an on-line computer were studied. Working with DCRT engineers and appropriate investigators, the specifications for an on-line system were established. A Request for Proposals was drawn up and sent to interested computer manufacturers. The responses from the manufacturers are now being evaluated.

Proposed Course of Study:

1. On Line Applications.
Systems are being designed for closed loop experimentation. Two areas are receiving immediate attention:

a) Computer control of visual stimuli in neurophysiological experiments for determining optimum responses during micro electrode recordings.

b) Computer control of multiparametric growth experiments with cariogenic streptococci.

2. Experimental Data Processing.
Further applications and improvements of computerized mathematical techniques to processing experimental data will be investigated. More sophisticated mathematical models will be developed to improve liquid scintillation counting programs. The investigation of better methods of resolving overlapping peaks in chromatographic data will be continued in collaboration with mathematicians of the Division of Computer Research and Technology.

3. Information Retrieval.
A system of computer programs is being developed to handle taxanomic data or general administrative information reports.

Part B not included

Report of the Laboratory of Microbiology
National Institutes of Dental Research
Summary Statement

Contemplation of shifting fashions in dental research indicates that it is timely to recollect continuing problems of the first importance, which have justified the principle premises on which programs of the Laboratory of Microbiology have contributed consistently and meaningfully during the past decade. Dental caries and chronic periodontitis continue throughout the world to constitute by far the major problems of dentistry. Despite much progress toward understanding their mechanisms, and despite development of at lease partially effective control measures, their elimination does not seem close to hand. As infectious diseases, both of these conditions result from complex interactions between an indigenous oral microbiota, the reactivity of the host tissues as influenced by innate and acquired resistance, and the external environment as exemplified by macro- and micronutrients and socio-economic parameters. A major commitment to oral microbiology therefore continues to be an essential component of dental research. But to be meaningful, study of oral microorganisms per se must be integrated into its ecological context, that is, the complex of host-parasite interactions. Such a comprehensive approach prospers best when infectious disease processes are investigated in organizational and geographic propinquity to such cognate areas as systematic microbiology, microbial physiology, immunology, and virology. Applied programs in collaboration with clinical, pathological, and epidemiological units are an essential component. But distinction between basic and applied studies eludes definition; they form a continuum, and dichotomous arrangements disadvantage both.

Our programs therefore have comprised studies directly concerned with mechanisms of experimental and clinical dental caries and periodontal disease; taxonomic studies aimed at better definition of members of the oral microbiota, to facilitate their identification and study of their ecologic habits; physiological studies focused on regulatory mechanisms controlling phenotypic expression of genotypic microbial potentialities, particularly intermediary carbohydrate metabolism ending in lactic and other acids, which are generally conceded to be the demineralizing agents in dental caries; immunological studies ultimately relevant primarily to periodontal disease, concerned on the one hand with local defenses peculiar to the oral cavity and on the other hand with immunopathological reactions affecting the oral tissues; and virological studies centering on factors influencing latent, persistent, and recurrent infections of the oral soft tissues. These investigations have entailed numerous collaborative projects with other laboratories and branches.

Dental Caries. Evidence continues to accumulate that in normal germbearing hamsters and rats the predominant cariogenic bacteria comprise a distinctive group of anaerobic streptococci. This year attention has been concentrated on the production by these bacteria of extracellular polysaccharide (dextran) specifically from sucrose. This polysaccharide seemed to contribute to development of caries because it was responsible for adherence

of the streptococci to teeth in the form of what is called dental plaque. Accordingly, it seemed logical to expect that application of a dextran-hydrolyzing enzyme (dextranase) should prevent plaque formation, remove preformed plaque, and reduce the initiation of caries by thus reducing the accumulation of cariogenic bacteria. Purified dextranase derived from a mold (Penicillium funiculosum) was made available through the cooperation of scientists at Merck and Company. When added to an in vitro culture system, the dextranase preparations prevented plaque accumulation and disintegrated preformed plaque. When the dextranase was incorporated in the diet and drinking water of hamsters on a cariogenic regimen, plaque did not accumulate and caries did not develop. These observations are most encouraging for a new approach to control of dental caries in humans. Field trials are pending to measure prevention of both plaque and caries by use of dextranase preparations.

Available evidence indicates that plaque accumulation requires dextran of high molecular weight. For example, incorporation of low molecular weight dextran in the diet and drinking water of hamsters prevents plaque and caries by what amounts to competitive or feed-back inhibition of the dextran-synthesizing enzyme, whereby production of high molecular weight dextran is suppressed. Likewise many dextran-forming strains of streptococci, and similar organisms of the genus Leuconostoc, are noncariogenic presumably because their dextran is not of the high molecular weight, insoluble, adherent type. These observations may point the way to an alternative method of caries control by preventing dextran synthesis.

Investigators of experimental caries in rats have long been puzzled, and often hampered, by the necessity of using only weanling animals, for the adults become insufficiently susceptible. A possible explanation now comes from collaborative studies with the University of Zürich on the teeth of gnotobiotic rats. When newly erupted, these teeth have hypomineralized areas at the bottom of the sulci where caries develops best in rats. As germfree rats mature, these areas become normally mineralized, and susceptibility to caries decreases. In rats monoinfected with a cariogenic streptococcus, however, normal mineralization of these areas does not occur; instead, progressive demineralization develops.

Use of a recently developed special transport medium, which keeps bacteria viable for many days but does not permit their multiplication, has greatly extended the range of our studies in epidemiological bacteriology. Samples of plaque were collected from groups of about 100 children each in (a) Cheektowaga, New York, a low-fluoride area with average caries incidence; (b) Charlotte, North Carolina, which has adequately fluoridated water and low caries incidence, and (c) two villages in Colombia, South America, with low fluoride water and a high caries rate. The samples were analyzed in Bethesda for cariogenic-type streptococci. The results confirmed previous preliminary findings indicating that the streptococci are more numerous and widespread where caries is more prevalent. However, repeated topical application of a fluoride gel by means of a mouthpiece during a two-year period (Cheektowaga study) did not reduce the streptococcal census.

Apparently these bacteria decline in a community only after a prolonged period of low-caries incidence.

This year the interplay of genetic and dietary factors in caries was studied in the classic Hunt-Hoppert caries-resistant and caries-susceptible strains of rats. These strains have been genetically stabilized by 25 years of selection and inbreeding. In these experiments, the microbial factor was equalized as well as possible by housing caries-active rats in the same cages with test animals. Differences in caries incidence could therefore be attributed to genetic and dietary factors respectively. Surprisingly, the Hunt-Hoppert strains have been classified as "resistant" or "susceptible" using only diets conducing to caries in the sulci of the teeth. When tested on a diet conducing to caries both in sulci and on smooth surfaces, the "resistant" strain did indeed develop few sulcal lesions, but it was highly susceptible to smooth surface caries. Paradoxically, the "susceptible" strain on the same diet developed less smooth surface caries than did the "resistant" strain. The total scores (sulcal plus smooth surface lesions) were the same in the two strains, but the ratios of the two types of lesion differed. At least part of these results can be explained by the fact that the diet conducing to smooth surface does so primarily by conducing to accumulation of plaque on the smooth surfaces, thereby in effect enhancing the bacterial challenge and also facilitating transmission of cariogenic organisms from animal to animal. On diets where the caries is confined to the sulci, the bacteria tend to remain impacted, and transmissibility is greatly reduced. Clearly resistance and susceptibility to caries are not absolute genetically determined attributes of the host but must be defined relative to the bacterial challenge and the modifying effects of different diets.

Periodontal Disease. Previous studies had shown that antigens, i.e., substances of high molecular weight, could be absorbed from the normal gingival pocket of rabbits, inducing antibody formation systemically and, locally, an allergic inflammatory reaction histologically very similar to that seen in human periodontal disease. It was suspected, however, that antigen absorption required a preliminary superficial break in the integrity of the crevicular epithelium. Using an improved irrigation procedure that does not per se irritate the pocket tissues, it has been shown that endotoxins and other antigens, which would be expected from bacteria in a periodontal pocket, do indeed not penetrate intact crevicular epithelium. When the pocket lining was ulcerated preliminarily, however, prolonged irrigation with concentrations of endotoxin as low as 0.01 μg per milliliter resulted in sufficient penetration to initiate inflammation in adjacent lymph nodes, with subsequent appearance of antibody-forming cells. Further study using direct injection of known quantities of endotoxin showed that with minute doses intramucosally, antibody-forming cells appeared only in the regional lymph nodes. Following similar minute doses intravenously, antibody-forming cells were found only in the spleen. With sufficiently large doses of endotoxin by either route, immune response was general and antibody appeared in the serum. These results suggest that with the small doses of bacterial antigens that are estimated to be available from bacteria in the gingival sulcus, both antibody response and immunopathological reactions may remain quite local.

Search for bacterial products that might initiate superficial alteration of crevicular epithelium continued to focus on ammonia and hydrogen sulfide. Detailed histopathological and electron microscopic description has been made of the effects of these substances on corneal epithelium. Using the innocuous irrigation system mentioned previously, it was found that irrigation of rabbit gingivae and lip mucosa with hydrogen sulfide in concentrations as low as 0.01 molar produced edema, erythema, and increased vascular permeability. Histopathological changes resembled those observed previously in cornea.

A filamentous bacterium designated as Odontomyces viscosus is the principal etiological agent of a periodontal pathosis in hamsters, which has been studied extensively in this laboratory and in the Laboratory of Histology and Pathology. A key feature of this pathosis is the accumulation subgingivally of large masses of gelatinous plaque. At least part of this plaque seems to be attributable to an extrabacterial polysaccharide (levan, comparable to the dextran produced by cariogenic streptococci), but the evidence indicated that something else was involved. Since in certain culture media O. viscosus produces a ropy, slimy growth not due to levan, such growth was analyzed for mucinoid factors, which have been identified as a complex of ribonucleic acid and protein. The possible role of such complexes in plaque prodromal to both periodontal disease and dental caries merits further study.

A number of bacterial strains resembling O. viscosus have been isolated from human gingival sulcal accumulations, but it has proved difficult to implant them in the mouths of hamsters, as a test of their ability to induce periodontal disease. Attention was turned to possible dietary parameters affecting implantation. Initially it was thought that a diet high in sucrose was essential for the hamster pathosis, but it has been found that other carbohydrates, such as glucose and starch, work as well. Preliminary data indicate that a starchy diet conduces to implantation of O. viscosus of human origin in hamsters. On this regimen, some of these strains have produced the characteristic periodontal pathosis in hamsters. This system promises to facilitate greatly a long-term study in progress of oral filamentous organisms and periodontal disease in a stable institutional population, where parameters such as diet and oral hygiene can be controlled.

Systematic Bacteriology. Last year we reported initiation of a long-term project in numerical taxonomy, primarily of oral bacteria, in collaboration with the Division of Computer Research and Technology, NIH, the trustees of Bergey's Manual of Determinative Bacteriology and microbiologists at Georgetown University, University of Maryland, and the American Type Culture Collection. Word of this project seems to have spread spontaneously through the bacteriological community and it has aroused what might well be called an exponentially rising curve of interest. With a view to making this program as comprehensive as possible, therefore, this year has been devoted to perfection of a data protocol defining some 250 parameters applicable to any of the known groups of bacteria. This phase of such a program is most critical, for if the most meaningful parameters are not selected, if

the questions are not phrased unambiguously, and if test conditions are not defined precisely, a consensus of respondents can not be obtained and mathematical analysis of the information is futile. From present indications, it does not seem fanciful to envision NIDR and NIH as a world center for general microbial taxonomy.

This laboratory has a continuing interest in species of Veillonella, a genus of gram negative anaerobic cocci, because they are the second most numerous bacteria in the human oral cavity. Study has now been essentially completed of organisms officially designated as Veillonella reniformis, but anomolous in a number of respects. Briefly, these organisms stain variably by Gram's method and they can utilize carbohydrate, though to a limited extent. Primarily they are fermenters of amino acids, principally glutamic acid, which they convert mostly to butyrate, acetate, carbon dioxide, hydrogen, and ammonia. When these data were assembled, it became evident that they fit equally well a group of presumably gram positive bacteria officially recognized as Peptococcus (Micrococcus) aerogenes. Tests of a number of strains of the latter, provided by other investigators, confirmed this similarity. The question of reaction to the Gram stain was resolved by electron microscopy in collaboration with the Laboratory of Histology and Pathology. These organisms have the multilayered outer cell wall characteristic of gram negative bacteria. Definitive taxonomic disposition of these bacteria is under negotiation by the several investigators concerned.

Bacterial Physiology. It is now possible to present a quite complete formulation of how lactic acid production is regulated in one species that has been under study, namely, Butyribacterium rettgeri. Regulation in this bacterium is essentially repressive, that is, it depends on the fact that the chief energy mediator of the cell (adenosine triphosphate, ATP) inhibits the enzyme lactic dehydrogenase (LDH), which irreversibly converts pyruvate to lactate. This inhibition provides the cell a mechanism to modulate the flow of carbon compounds according to the demand for biosynthesis and the available supply of ATP needed to energize biosynthesis. Briefly, when ATP is low, carbohydrate is glycolyzed to lactate with concurrent increase in ATP but not of pyruvate for biosynthesis. With accumulation of ATP, LDH is inhibited, and continued glycolysis supplies pyruvate rather than lactate. Pyruvate and ATP are then consumed in the synthesis of amino acids and other structural components, glycolysis to lactate is resumed, and so on. Experiments with purified LDH from B. rettgeri, too detailed to recount here, have made possible formulation of a molecular model explaining the inhibition of LDH by ATP. Essentially, the catalytic function of this enzyme is regulated by means of alterations of its three-dimentional structure which are mediated by interactions with ATP or its coenzyme. In the normal enzymically active configuration, the enzyme is in reversible combination with the necessary coenzyme and can effect conversion of pyruvate to lactate. In allosteric configuration, the enzyme combines reversibly with ATP, and combination with coenzyme is impossible.

In streptococci, the regulation of lactate production is positive in character. That is, LDH in these organisms is normally inactive and requires specific activation by critical levels of fructose diphosphate (FDP), an early intermediate in the glycolytic scheme. (coincidentally, FDP represses a lactate degrading system.) Present evidence indicates that FDP combines with LDH and induces a configurational change to create a site on the LDH molecule suitable for combination both with coenzyme and substrate (pyruvate).

Rather different regulatory mechanisms seem to operate in streptococci grown on substrates other than glucose, notably malate. In this case, the malic enzyme, which initiates utilization of malate by the so-called shunt pathway, is inhibited by ATP, FDP, and 6-phospho-gluconate. Though many details need to be determined, these facts indicate that in this case regulation is essentially repressive, as it is in B. rettgeri.

Immunology. Research in the Immunology Section concentrated mainly on the role of serum complement (C') as a mediator of biological effects of bacterial endotoxins and on factors controlling the phenomenon of immunological tolerance.

Collaborative studies with investigators at the Johns Hopkins Medical School defined the reactions of endotoxin with C' in normal sera and showed that at least two of the biological actions of endotoxin are mediated by this reaction. Classically, C' has been considered as an adjuvant to the combination of antigen with antibody, with "fixation" of C' and subsequent manifestations such as lysis of cells bearing the antigen, enhancement of phagocytosis, and production of anaphylactic reactions. Recent studies, however, have shown that complement mediates a variety of physiological phenomena not involving any antigen-antibody reaction. In the case of endotoxin, incubation with fresh normal sera results in the utilization of components C'3,5,6,7,8 and 9 but not of C'1,4, and 2. In the clasical reaction with antigen-antibody complexes, fixation of C'1,4, and 2 is a necessary forerunner of the reactions with the remaining 6 components, in the order indicated. The consumption of (C'3,5,6,7,8, and 9) by endotoxin is paralleled by a release of anaphylatoxin, a factor which in vivo brings about smooth muscle contraction and increased capillary permeability, presumably by releasing histamine. The reaction of endotoxin with normal serum also liberates a chemotactic factor which causes polymorphonuclear leukocytes to migrate into its vicinity. These several reactions are directly applicable to explain the production of acute inflammation by endotoxin, which is the ultimate concern here. It remains to ascertain whether under these conditions anaphylatoxin and chemotactic factor result from breakdown of endotoxin by complement components, or vice versa.

Increasing general interest in the feasibility of tooth transplantation prompted initial studies of some transplantation phenomena. As is well known, unless the transplanted tissue is autogenous or isogeneic, the recipient rejects it sooner or later by an immunological response of the delayed, cell-mediated hypersensitivity type. Accordingly, much effort has gone into the use of immunosuppressive agents, of which antilymphocyte

serum (ALS) currently seems promising. Unfortunately, ALS itself is foreign to the recipient and soon removed by immune elimination. The question was asked whether the immunosuppressive action of ALS could be prolonged if the recipient had been made immunologically tolerant. ALS was prepared by injecting mouse lymphocytes into rabbits. This ALS was administered (a) to untreated mice and (b) to mice rendered immunologically tolerant by prior injections of normal rabbit gamma globulin. Skin homografts were then applied to the mice. Those on the immunologically tolerant animals persisted much longer, thus confirming the test hypothesis.

Previous collaborative studies with the Virology Section had shown that chronic virus infection, using lactic dehydrogenase virus (LDV), acted as an immunological adjuvant in mice, for it raised the normal gamma globulin level and enhanced the antibody response to a heterologous antigen. Extension of the experiments has shown that such virus infection has the opposite effect on a cell-mediated immunological reaction of the delayed hypersensitivity type, namely, the so-called graft vs. host reaction. In this experiment, cells extracted from mouse spleens are injected into other mice. Ordinarily, the recipients develop a marked enlargement of the spleen within about 48 hours. The reaction can be quantitated simply by removing and weighing the spleens. In mice infected with LDV, such splenomegaly did not develop, indicating that the virus acted as an immunosuppressive agent.

Virology. As a corollary to the foregoing experiments, the effect of virus infection on immunological tolerance was studied. Mice were inoculated with doses of human gamma globulin (HGG) such that (a) they did not develop antibody to HGG and (b) they did not develop antibody to subsequent doses of HGG which would induce antibody in untreated mice. That is, the mice were rendered immunologically tolerant to HGG. When the mice were subsequently infected with LDV, however, this tolerance was "broken" and antibody was produced forthwith. Other means, such as injection of endotoxin, have been shown to break tolerance but this is the first time a virus infection has been shown to do so. These observations raise the question whether some virus infections might not convert the host's normal tolerance of his own tissues to auto-antibody formation, and thus play a trigger role in some auto-immune diseases.

Study of the paradoxical coexistence of infective virus and antiviral antibody in the serum during LDV infection has established the principle that a virus can remain infective in a virus-antibody complex, that is, some antibody combines with virus without neutralizing it. Such virus is said to be sensitized, and it is demonstrated by the fact that it is neutralized by anti-gamma-globulin homologous to the antiviral antibody (by anti-antibody, as it were). On the other hand, sensitized virus is markedly resistant to neutralization by addition of more antiviral antibody. This phenomenon affords an explanation for a number of viral infections that persist or recur in the face of antibody, such as human serum hepatitis, lymphocytic choriomeningitis, and herpes labialis. Viral sensitization seems to be a general phenomenon. We demonstrated it also with herpes simplex virus (HSV) and a number of laboratories subsequently have demonstrated in both *in vitro* and *in vivo* with a variety of viruses.

We have directed attention also to refinement of the "anti-gamma" neutralization test with sensitized virus with a view of its use as a highly specific, highly sensitive serological tool. Details are given in the project report of the Virology Section. Briefly, conditions have been defined for optimal degree of sensitization, for appropriate concentrations of anti-gamma, and for removal of excess gamma globulin in the virus-antivirus mixture. A modification of the standard serum-dilution neutralization test permits quantitation of the resistance of sensitized virus to further neutralization by antiviral antibody. With a virus that can be quantitated by a plaque technique, such as HSV, such resistance to neutralization can be measured precisely by the reduction in its rate of neutralization (neutralization inhibition kinetics). Using these methods, it was shown with both LDV and HSV that resistance to further neutralization by antiviral antibody correlates closely with degree of sensitization.

With these methods it has become feasible to use sensitized virus as an indicator for detecting specifically and titrating different classes of immunoglobulin, such as gamma G, M, or A, allotypic immunoglobulin, and active fragments of immunoglobulin derived enzymically. For example, using HSV sensitized with human sera and applying the anti-gamma test with anti-human gamma G and anti-human gamma M, respectively, it has been found so far that the anti-HSV is always of the Gamma G type, though the antibody formed first after a primary infection was expected to be of the gamma M class. Conversely, virus sensitized with antiviral antibody of a given immunoglobulin class can be used to detect and titrate homologous normal anti-immunoglobulin.

Aside from its potentiality for a renaissance in the theory of the viral neutralization reaction, the possible practical application of viral sensitization and of the anti-gamma neutralization test are too numerous to detail here. One of the most obvious is the diagnostic detection and titration of anti-viral antibody in weak sera, or in sera whose antibody sensitizes but does not neutralize. Traditionally, neutralization of virus by dilutions of serum less than about 1:4 has been regarded as non-specific and insignificant. The anti-gamma test in effect increases the titer of a serum by as much as 64-fold. Workers elsewhere are already utilizing this leverage to demonstrate antibodies to adenoviruses and arboviruses in sera previously diagnosed as negative.

Serial No. NIDR-9 (59)
1. Microbiology
2. Gnotobiotic
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Gnotobiotic Studies of Problems Relating to Oral Disease

Previous Serial Number: NIDR-2

Principal Investigator: Dr. R. J. Fitzgerald

Other Investigators: Dr. R. H. Larson
Dr. P. H. Keyes
Dr. R. J. Gibbons (v.s.)

Cooperating Units: Dental Research Institute, University of Zurich; Microbiology Research Laboratory, Merck Institute

Man Years:

Total:	6-1/4
Professional:	1
Other:	5-1/4

Project Description:

Objectives:

This activity is a continuing project in which germfree and gnotobiotic animals are used in the study of diseases of the oral cavity.

Methods Employed:

Microbiological and biochemical laboratory technics are used to study microorganisms suspected of involvement in oral diseases Germfree, conventional and limited flora animals are used to investigate the role of these organisms in disease processes.

Major Findings:

1. Maturation of hypomineralized areas in molars of gnotobiotic rats (with Dr. König, University of Zurich). Newly erupted rat molars show hypomineralized areas of enamel, particularly at the base of the fissures, the site most susceptible to caries. In germfree animals it was found that the degree of hypomineralization detectable by stainability with silver nitrate begins to decrease about 20 days after eruption of the tooth and maturation is essentially complete after 60 days. In gnotobiotic rats monoinfected with a cariogenic streptococcus and maintained on a high sucrose diet, maturation of the hypomineralized areas was completely prevented due to the superimposed effects of carious demineralization. The results are consistent with the clinical impression that newly erupted teeth are especially susceptible to caries and suggest that one reason for this may be the incompletely mineralized state of the enamel in some areas of these teeth. The observation that mineralization increases with exposure to a non-caries conducive oral environment may explain why teeth become more resistant to caries the longer they exist in such an environment.

2. Inhibitions of plaque formation and caries in animals treated with dextranase (with Dr. Keyes and Dr. Stoudt, Merck and Co.). The streptococci of human or animal origin which have been found to induce caries in rodents, characteristically produce large amounts of extracellular insoluble dextran when metabolizing sucrose. This dextran has been associated with the ability of these organisms to form microbial plaque deposits on teeth of humans or animals. If the hypothesis is correct that dextran mediated plaque deposition is a key factor in caries induction by these organisms, then agents which can destroy or prevent the formation of dextran could be effective in caries and plaque control. One such agent, the enzyme dextranase was administered to albino hamsters which had been infected with a cariogenic dextran-producing streptococcus and maintained on a high sucrose diet. Depending on the time at which dextranase was administered either the complete dentition or those teeth which erupted after dextranase administration began could be protected from caries. Coronal dental plaque deposits were removed and the progress of caries which had begun in teeth prior to dextranase administration was retarded.

3. Inhibition of plaque formation and caries in animals by low molecular weight dextrans (Dr. Gibbons and Dr. Keyes). It has been found that low molecular weight dextrans (ca 20,000 M.W.) will inhibit the synthesis of insoluble high molecular weight dextrans by the enzyme dextran-sucrase of cariogenic streptococci. If high molecular weight dextrans are responsible for plaque formation by cariogenic streptococci then conceivably the administration of low molecular weight dextran to animals infected with these microorganisms should inhibit both plaque formation and caries. It was found that both plaque formation and the development of caries could be prevented in hamsters infected with dextran-producing cariogenic streptococci when the high sucrose diet on which they were maintained was supplemented with 16% of dextran of molecular weight of 20,000.

4. Dextran-producing streptococci and caries in gnotobiotic rats. The ability to produce extracellular dextrans from sucrose is a property which is common to several species of streptococci including Leuconostoc mesenteroides, Streptococcus sanguis and Streptococcus bovis as well as the cariogenic streptococcal strains isolated in our laboratory. Several strains of the above mentioned species have now been implanted in gnotobiotic rats but have failed to induce progressive dental caries lesions. Since both S. bovis and S. sanguis have approximately the same acid producing potential as the cariogenic strains, other factors in addition to acid production and dextran production appear to be essential for cariogenicity. One possibility which is now under study is that types of dextran produced by the organisms may vary in molecular size and adhesive properties from strain to strain with consequent effects on their ability to form plaque deposits on teeth.

Significance to Dental Research:

Caries susceptibility in the rodent model system which we employ may be influenced on the part of the host by the degree of maturation of the enamel. As far as the attacking organisms are concerned acidogenisis and dextran production from sucrose are common characteristics of all cariogenic streptococci, but some organisms which are not cariogenic also exhibit these properties. Since the enzyme dextranase, which specifically attacks dextran, can inhibit plaque formation and smooth surface caries in hamsters, dextran must now be accorded a key role in plaque formation by these cariogenic streptococci. The possibility therefore arises that cariogenic streptococci may produce types of dextrans which differ in physical or chemical properties from those elaborated by non-cariogenic dextran formers.

Proposed Course of Project:

Future studies will be conducted in the three areas.

1. Testing of dextranase for possible utility in controlling plaque and caries in humans.

2. Basic studies to determine the metabolic basis for the cariogenic ability of the caries conducive streptococci.

3. Epidemiological studies to determine the geographic distribution of potentially cariogenic streptococci in humans.

Part B:

Publications:

1. Fitzgerald, R. J., Spinnell, D. M., and Stoudt, T. H: Enzymatic removal of artificial plaques. Arch. oral Biol. 13: 75, Jan. 1968.

2. Fitzgerald, R. J., Keyes, P. H., Stoudt, T. H., and Spinell, D. M: The effects of a dextranase preparation on plaque and caries in hamsters, a preliminary report. J. Amer. Dent. Assn. 76: 301, Feb. 1968.

Serial No. NIDR-10 (63)
1. Microbiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Studies of Culture Media for the Mass Cultivation of Representatives of the Genera Treponema and Borrelia. The Use of Bovine Serum Fraction for Growth Initiation of Spirochetes.

Previous Serial Number: NIDR-4

Principal Investigator: Dr. E. G. Hampp

Other Investigators: None

Cooperating Units: American Dental Association

Man Years:

Total:	1-1/4
Professional:	1
Other:	1/4

Project Description:

Objectives:

1. To determine the factors present in ascitic fluid and serum products that are essential for growth of spirochetes in a medium that is otherwise nutritionally complete.

2. To study the various commercially available components of bovine serum for growth enhancement of the spirochetes in broth media.

3. Comparison of ascitic fluid, bovine serum albumin, Dubos oleic acid complex and Middlebrooks OADC enrichment for growth initiation of the spirochetes.

Methods Employed:

A medium prepared from dehydrated products and designated as BHI was used in previous studies as an agar medium both for isolation of oral spirochetes and also for the cultivation of new and old strains of a variety of these microorganisms. The BHI agar medium containing 0.1% reduced glutathione was also employed previously to assay the growth potential of bovine serum fractions for certain strains of spirochetes; it was ideal for this purpose since no growth occurred

unless ascitic fluid or serum was incorporated in the finished medium. During this period, the BHI medium was prepared as a broth containing 0.1% glutathione and was used for the evaluation of serum fractions as well as other substance for initiation of spirochetal growth. In these experiments two strains of oral treponemes (FM, MRB), two strains of Borrelia vincentii (CON, N9) and the Reiter treponeme were used as representatives of the spirochetal collection. These organisms had been previously adapted to grow in Huntoon's hormone agar containing 0.1% glutathione but no ascitic fluid or serum supplement. As a source of inoculum for broth culture experiments, the organisms were grown free of ascitic fluid in Huntoon's broth containing 0.1% glutathione. One ml quantities spirochetal containing broth culture was used for an inoculum in 18 ml experimental medium in screwtop test tubes. The bovine serum fractions (NBC) were used in 0.1% final concentration in the experimental medium and included beta lipoproteins FR. III 0, FR IV 1 and FR IV 4, also, glycoprotein FR VI, beta globulin FR III, gamma globulin FR II, globulin FR IV 7, alpha globulin FR IV 1, and beta globulin FR IV 4. Other test substances included hog gastric mucin (Wilson, 701W), sodium glucuronate, D(+) glucosamine-free base, acetyl glucosamine and D(+) glucosamine; these were employed in a final concentration of 0.4% in the finished BHI medium. Each of these substances was filter sterilized and autoclaved (10 ml amounts, 3 min. 121°C). However, autoclaved samples of glycoprotein FR VI and gamma globulin FR II could not be used since they were coagulated by heat. In addition, bacteriologic grade bovine serum albumin FR V, crystalline bovine albumin FR V, Dubos oleic acid albumin complex and Middlebrook's OADC enrichment were used individually in 0.5% concentration in the BHI medium. These serum products were obtained commercially in a sterile condition with the exception of the crystalline albumin, and it was filter sterilized in the laboratory. Control cultures consisted of BHI medium with and without an asitic fluid supplement. Growth estimation was determined by cell enumeration using a Petroff-Hauser counting chamber.

Major Findings:

The spirochetes are fastidious microorganisms requiring a complex medium for growth, and, in addition, having an absolute requirement for ascitic fluid or serum products which further complicates their study. However, certain strains of spirochetes may be adapted to grow to a limited extent in Huntoon's agar and broth in the absence of a serum additive. This finding may in part be due to the presence of an alcohol precipitable substance that was previously demonstrated in a low heat veal heart infusion and was shown to substitute to a certain degree for ascitic fluid. Recent studies demonstrated that spirochetes could be grown in BHI agar only if it contained ascitic fluid, serum products or fractions of bovine serum. Therefore, it was advisable to pursue further these findings in BHI broth medium. When grown in the experimental medium with ascitic fluid, the average

counts for the five strains of spirochetes were as follows: FM, 1.35×10^9; MRB, 1.5×10^9; N9, 6×10^8; CON, 4.4×10^8, and ER 5.35×10^8. The BHI broth control cultures without ascitic fluid did not show growth of any of the spirochetes. However, when the beta lipoproteins FR III 0, FR IV 1 and FR IV 4, both filtered and autoclaved, were used in the basic medium in a final concentration of 0.1%, it was found that all strains of spirochetes grew at a level between 40-60% of the ascitic fluid controls. Glycoprotein FR VI initiated growth of all strains of spirochetes but it was 20-30% of that of the control cultures. In this experiment, only the filter sterilized material was employed since heat coagulated the solution to be tested. When the filter sterilized and autoclaved solutions of bovine globulins, onsisting of beta globulin FR III, gamma globulin FR II, globulin FR IV 7, alpha globulin FR IV 1, and beta globulin FR IV 4 were employed in the BHI broth medium at a final concentration of 0.1%, they elicited a growth response of all the strains of spirochetes that varied between 50-70% of that of the control cultures. The autoclaved sample of gamma globulin FR II was not used since it coagulated on autoclaving. When gastric mucin was employed at 0.4% concentration in the BHI broth it supported the growth of all 5 strains of spirochetes at about 20-30% of that of the control cultures. However, when sodium glucuronate, D(+) glucosamine free base, acetyl glucosamine and D(+) glucosamine were employed at the 0.4% level in the growth medium, both filter sterilized and autoclaved, they had no effect on growth initiation of any of the 5 strains of spirochetes. It was also found that the bacteriologic grade of bovine serum albumin FR V was superior to the highly purified crystalline bovine serum albumin FR V as well as Dubós oleic acid complex and Middlebrook's OADC enrichment. Also, compared with the ascitic fluid controls, the bacteriologic grade serum albumin was 70-80% as effective in growth initiation of the spirochetal strains. The mode of action of the bovine serum fractions for spirochetal growth is unknown and needs further study. As previously pointed out, the lipoproteins of serum contain considerable amounts of cholesterol, phosphatids and fatty acids and it is possible that the protein moiety may detoxify certain of the lipids and release them as needed by the spirochetes. Oyma et al. (J. Bacteriol. 65: 1953) have shown that serum albumin serves the sole purpose of detoxification of oleic acid and releases the material as needed by the Reiter treponeme. The bovine globulin fractions also contain varying amounts of lipid, with the exception of beta globulin IV 4, which is virtually free of this material. Despite its freedom from lipid, however, the later fraction still elicited good growth of the various spirochetes. There is also a possibility that traces of serum albumin are carried over in the lipoproteins and the globulins, which may in part account for their mode of action.

Significance to Dental Research:

The oral spirochetes constitute a complex group of microorganisms about which little is known concerning their metabolic requirements. They will only grow in complex media and require as well supplements of ascitic fluid or serum products which further complicates the exploration of their biochemical processes. The lack of knowledge concerning the mode of action of ascitic fluid has hampered progress on the development of a chemically defined growth medium and a means of identification and classification of these microorganisms on a sound basis. It is foreseeable when these obstacles are surmounted that such studies may contribute to the elucidation of their relationship to acute necrotizing ulcerative gingivitis since they have long been implicated in this disease process.

Proposed Course of Project:

1. Studies will be continued on the absolute requirement of the spirochetes for ascitic fluid or serum products for growth initiation in complex media.

2. Since serum fractions have been shown to initiate growth of the spirochetes in place of ascitic fluid, a variety of similar materials such as myosinogen, oxoglobulin, edestin, amadin, legumin, excelsin, protosoy, zein, and other purified proteins may be useful in gaining some insight into this problem.

3. The bovine serum fractions may possibly be further purified by charcoal treatment at low pH and with virtually complete removal of fatty acids (Chen, J. Biol. Chem., 242: 1967) for further study of their effect on growth initiation of the spirochetes.

4. A partially defined medium (Nevin and Hampp, J. Bacteriol. 78: 1959) may be of use in these studies with serum fractions since it will permit addition and deletion of certain defined components of the medium.

Part B Not included

Serial No. NIDR-11 (65)
1. Microbiology
2. Gnotobiotic
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Relationship of Specific Oral Bacteria to Dental Caries and Periodontal Disease

Previous Serial Number: NIDR-5

Principal Investigator: Dr. H. V. Jordan

Other Investigators: Dr. P. H. Keys, Dr. H. R. Englander

Cooperating Units: None

Man Years:

Total:	2-1/4
Professional:	1
Other:	1-1/4

Project Description:

Objectives:

1. To study the relationship between certain oral streptococci and dental caries in experimental animals and to apply this knowledge to a study of the disease in humans.

2. To investigate the mechanism by which the filamentous microorganism Odontomyces viscosus induces periodontal disease in experimental animals and to extend the studies in organisms of this type to periodontal disease in humans.

Methods Employed:

Specific strains of bacteria are studied using standard bacteriological and biochemical techniques. Certain aspects of the diseases are simulated in a laboratory model under controlled conditions. Included are in vitro models, as well as gnotobiotic rats in the experimental hamster model.

Extensive use is made of infecting bacteria "tagged" with antibiotic resistance in order to follow their implantation and colonization in animal studies.

Part A (continued)

Major Findings:

1. Dental caries and specific streptococci. Microbiological surveys were conducted on a number of population groups in order to establish the presence and distribution of caries inducing streptococci in humans. Relative numbers of streptococci similar to known caries inducing types were recorded in plaque samples from school children, aged 12-14 years using the method described in the 1967 project report. Four population groups were sampled: 111 children in Cheektowaga, N. Y. (0.3 ppm NaF) with an average of 6.5 DMFT; 97 children in Charlotte, N. C. (1.2 ppm NaF) with an average of 2.3 DMFT and 104 children from 2 different villages in Colombia, South America with averages of 12.4 and 4.1 DMFT. Approximately 60 percent of the children in the Cheektowaga and Charlotte groups received daily applications of a NaF gel applied topically to the teeth in fitted mouthpieces. The Cheektowaga children had received over 200 fluoride treatments over a period of 19 months and plaque samples were taken 11 months after the treatments had stopped. The Charlotte children had received a total of 59 treatments over a period of 5 months and the experiment was still in progress when the plaque samples were taken.

   The Colombian populations were of interest because of a known difference in caries experience which could not be explained on the basis of dietary differences or fluoride intake.

   Specific caries-inducing streptococci were detected in 70 percent of the Cheektowaga subjects, in 30 percent of the Charlotte subjects, and in 69 and 59 percent of the subjects respectively, in the high and intermediate caries groups in Colombia. In the Cheektowaga and Charlotte groups the incidence and numbers of the specific streptococci were essentially the same for the fluoride-treated children and the untreated controls.

   Ninety-nine strains of the caries-inducing types of streptococci were isolated from the 4 population groups and characterized biochemically. All fermented mannitol and most strains fermented sorbitol. All strains formed a very adherent growth when cultured in sucrose broth. One hundred and two strains representing all other types of streptococci cultured from the plaque were universally negative in these characteristics. Dental caries was induced in gnotobiotic rats and in the conventional hamster model using 8 representative strains of the caries-inducing types.

   It was concluded that these specific bacterial types are widespread in various human populations, but they may occur in high numbers sometimes constituting 50 percent or more of the plaque streptococci, and their presence correlates with caries experience

Part A (continued)

on a group basis. Caries-free individuals were usually free of these types of bacteria. In a selected group of school children observed over a one year period, a strong correlation was noted between the presence and numbers of the caries-inducing streptococcal types and the appearance of a new smooth surface carious lesions. Repeated topical applications of a NaF gel had little immediate influence on the presence or relative numbers of caries-inducing streptococci in dental plaque.

In cooperation with the Epidemiology Group at NIDR a project was initiated using personnel at the Coast Guard Station on Governors Island in New York. The bacteriological aspects of this study are designed to determine the incidence of the specific caries-inducing streptococci in nursery school children, 2-4 years of age. Since a large percentage of these children are caries-free and their oral flora may be less complex than that of adults, this population is considered an excellent model in which to study the bacteriology of dental caries. Preliminary studies have indicated that the specific organisms are present in only 30 percent of these children. Bacteriological examinations will be conducted periodically in order to correlate caries onset and progression with the appearance of caries-inducing streptococci. The population is composed of a large number of sibling groups which provides an opportunity to observe transmission patterns of the organisms within family groups.

2. Relationship of Odontomyces viscosus and other filament-forming bacteria to periodontal disease. Original studies which established the etiologic relationship between O. viscosus and periodontal disease in hamsters were carried out using a sucrose-containing diet. As described in the 1967 progress report O. viscosus produces an extracellular levan from sucrose which may be of some significance when the organisms colonize the oral cavity of hamsters fed a sucrose diet. However, previous work has shown that the sucrose component of the diet is not vital to the initiation and progress of the disease. The infecting organism becomes implanted and induces the typical disease syndrome when the hamsters are fed a diet containing rice flour or corn starch in place of sucrose. Studies are now underway which will attempt to define the dietary conditions controlling the implantation and colonization of O. viscosus and related bacteria. Groups of hamsters infected with a streptomycin-resistant strain of O. viscosus are fed a diet in which the sucrose is replaced with different monosaccharides. Periodic cultures are taken to follow the course of the infection.

Part A (continued)

O. viscosus grows as a stringy viscous culture in the presence of glucose. A high molecular weight material can be collected by alcohol precipitation of viscous broth cultures. Chemical analysis indicates the presence of a nucleic acid-protein complex in this material. Continuing studies will investigate the relationship between this phenomenon and the plaque forming ability of the organism.

Cooperative studies have recently been initiated with personnel at the Lincoln State School, Lincoln, Illinois, an institution for mental defectives. The high prevalence of periodontal problems in this population makes it an ideal source of material to study bacteriological factors in periodontal disease. Samples are collected at the Lincoln School and sent to Bethesda for examination, using the transport medium and method described in last year's report. A variety of filamentous bacteria have been isolated and are currently being tested for their ability to form gingival plaque and periodontal pathology in hamsters and gnotobiotic rats.

Significance to Dental Research:

This work should be considered as an attempt to project the knowledge gained from animal experiments to a study of dental caries and periodontal disease at the human level. Population studies of the type described will yield necessary information about the relative proportions of the caries-inducing streptococci in different kinds of plaque, their correlation with different types of dental caries and their distribution in different population groups.

Studies on periodontal disease are expected to describe factors of importance which influence the ecology of the oral flora as it relates to periodontal disease. Present studies are centered on the filaments because they are known to colonize in the gingival crevice. Current studies with filaments isolated from humans and tested under different dietary conditions may lead to a useful animal model system for selecting organisms involved in human periodontal disease. Studies of extracellular materials produced by these organisms will help to explain some of the mechanisms of plaque formation.

Proposed Course of the Project:

Distribution patterns of the caries-inducing streptococci in various human populations around the world have been established as a result of the epidemiological surveys described. Future studies of this type will be concerned with establishing the

Part A (continued)

origin and transmission of these organisms within population groups. Studies of a more intensive nature on selected populations will be designed to examine the role of these specific bacteria in the initiation and development of particular types of carious lesions. The influence of certain dietary factors on the implantation and persistence of these organisms will also be investigated. Certain aspects of these studies on human populations, such as dietary variables will be duplicated in animal models for study under controlled conditions.

Continuing studies with the filament-forming bacteria will be oriented toward experiments on the mechanism by which these organisms become established in the gingival sulcus and induce periodontal disease. The parameters governing this pathogenicity are not well defined as are the disease parameters of dental caries. Future studies will attempt to describe dietary factors of this type. Isolation and testing of filamentous bacteria from the human oral cavity will continue in order to establish the pathogenic potential of organisms of this type in periodontal disease. One of the objectives will be to determine if many types or a single specific type of filament-forming organism is involved. Future studies will be broadened to include other organisms in addition to the filament.

Part B Publications

1. Howell, A., Jr. and Jordan, H. V.: Production of an extracellular levan by Odontomyces viscosus. Arch. Oral Biol., 12: 571-573, 1967.

2. Jordan, H. V., and Krasse, B.: A method for sampling human dental plaque for caries-inducing streptococci. Arch. Oral Biol., in press.

3. Krasse, B., Jordan, H. V., Edwardsson, S., Svensson, I., and Trill, L: The occurence of caries-inducing streptococci in human dental plaque material. With special reference to the frequency of caries in selected groups of persons. Arch. Oral Biol., in press.

Serial No. NIDR-12 (61)
1. Microbiology
2. Gnotobiotic
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Role of Genetic and Environmental Factors in Experimental Dental Caries

Previous Serial Number: NIDR-7

Principal Investigator: Dr. R. H. Larson

Other Investigators: Dr. R. J. Fitzgerald

Cooperating Units: University of Zürich, Zürich, Switzerland
University of Hawaii, Honolulu, Hawaii

Man Years:

Total:	2-1/4
Professional:	1
Other:	1-1/4

Project Description:

Objectives:

This is a continuing project to evaluate the separate and interrelated roles of host animal, oral flora and dietary challenge on the development of caries in laboratory rodents.

Methods Employed:

Conventional methods for animal experimentation have been used. Both the levels and patterns of caries activity have been studied for several strains of rats and mystromys with variations in oral flora and dietary challenge.

Major Findings:

Studies of the interrelationship between host factors, diet and bacterial infection have been continued. Animals which had originally been considered caries resistant were shown to be highly caries active when challenged by certain combinations of diet and bacterial infection.

Part A (continued)

A. Hunt-Hoppert rats. For the past 25 years workers at Michigan State University have bred and studied two strains of rats which they designated as caries-susceptible (Ca-S) and caries-resistant (Ca-R). Throughout these studies the diet used was of the type which is associated with caries in the sulci only. Under these conditions, there was no increase in caries as a result of transmission of flora, and throughout the years of study the two strains of rats continued to show the widely different levels of caries development.

When different dietary-bacterial challenges were provided, a better understanding of the host differences became evident. (1) When Ca-S and Ca-R rats were challenged with a coarse particle corn diet, caries development was essentially the same as that in the Michigan studies. The total number of carious enamel areas (CEA) was twice as high for the Ca-S (33.0) as for the Ca-R (16.7), the lesions were almost exclusively in the sulci and the infected group showed no increase over the uninfected. (2) The animals fed a fine particle, high sucrose Diet 2000, developed lesions on all surfaces of the teeth. The uninfected Ca-S animals developed twice as many CEA (51.5) as the Ca-R (22.4), with 38.6 vs.18.6 in the sulci and 12.5 vs. 3.8 on the smooth surfaces. Both infected groups showed increased activity over the controls and the Ca-S and Ca-R developed an equally high total number of CEA (71.2 vs. 71.4). However, the infected CA-S developed almost twice as many CEA in the sulci as the Ca-R (42.7 vs. 23.7) and only half as many on the smooth surfaces (28.5 vs. 47.7).

The results of these studies show that the Ca-R animals are not actually caries resistant, but were caries inactive in early studies because the challenge was limited to the sulci only. When a suitable combination of diet and flora was provided, the Ca-S and the Ca-R animals appeared to be equally susceptible, but the pattern of lesion distribution differed for the two strains.

B. The white-tailed rat (Mystromys albicaudatus). It was suggested by Ockerse in 1953 that the white-tailed rat is a suitable animal for experimental caries studies. However, the only animals in which he reported the development of lesions were offspring of mothers which were maintained on a high sucrose diet during pregnancy and lactation. The role of an organism, not native to this species, was clearly demonstrated in an experiment in which they were fed Diet 2000. The uninfected control animals averaged less than 4 carious lesions each, whereas those infected with a streptococci of human origin (K1-R) averaged 27 lesions each. This organism was already known to be caries conducive in rats, hamsters and gerbils.

Part A (continued)

This study shows again that animals may be caries inactive only because the specific type of organisms necessary for the caries process is not present. Since this work was done in conventional animals it is not known what other organisms, native to the host, may have participated in the caries process.

Significance to Dental Research:

Any advancement in the knowledge of factors associated with experimental caries should lead to a better understanding of the human disease process and to the development of methods for its control.

Proposed Course of Project:

With each advance in the understanding of the influence of various factors in the experimental caries process it becomes more evident that the effect is mediated by its influence on the bacterial challenge. Thus, it would appear that any real advance in the eradication of this disease will require a more complete knowledge of the microbiota involved in the process. The inability, so far, to produce the rampant disease in gnotobiotes which can be produced in conventional animals suggests that a combination of several organisms may be required for the process. However, the finding that streptococci K1-R had an almost catalytic effect in the development of caries in several species of animals suggests the possibility that a limited number of organisms may play an essential role. If such is the case, the identification of these organisms, their nutritional requirements, and a knowledge of their metabolic processes and byproducts should lead to a means by which the disease could be radically reduced.

Part B Publications:

1. Larson, R. H. and Keyes, P. H.: The influence of reduced salivary flow on the intensity of the cariogenic challenge. Helv. odont. Acta, 11: 36-43, 1967.

2. Fitzgerald, R. J. and Larson, R. H.: Age and caries susceptibility in gnotobiotic rats. Helv. odont. Acta, 11: 49-52, 1967.

3. Larson, R. H., Theilade, E., and Fitzgerald, R. J.: The interaction of diet and microflora in experimental caries in the rat. Arch. oral Biol., 12: 663-668, 1967.

Part B (continued)

4. Chung, C. S. and Larson, R. H. Factors and inheritance of dental caries in the rat. J. Dental Res., 46: 559-564, 1967.

5. Larson, R. H. and Goss, B. J.: Diet as a limiting factor in the transmissibility of caries activity between rats of different strains. Arch. oral Biol.,12: 1085-1094, 1967.

6. Frostell, G., Keyes, P. H. and Larson, R. H.: Effect of various sugars and sugar substitutes on dental caries in hamsters and rats. J. Nutrition, 93: 65-76, 1967.

7. Chung, C. S., Larson, R. H., and Goss, B. J.: Perinatal and growth associated factors influencing dental caries in rats. J. Dental Res., 47: 139-141, 1968.

8. Larson, R. H., Keyes, P. H. and Goss, B. J. The development of caries in the Hunt-Hoppert caries-susceptible and caries-resistant rats under different experimental conditions. J. Dental Res., in press.

9. Larson, R. H. and Fitzgerald, R. J.: Caries development in the African white-tailed rat (Mystromys albicaudatus) infected a streptococcus of human origin. J. Dental Res., in press.

10. Larson, R. H.: Tooth age and caries susceptibility. In Harris, R. E. (Ed.): The Art and Science of Dental Caries Research. New York, N. Y., Academic Press, in press.

Serial No. NIDR-13 (66)
1. Microbiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Physiology and Regulation of Metabolic Processes in Lactic Acid Bacteria

Previous Serial Number: NIDR-8

Principal Investigator: Dr. J. P. London

Other Investigators: None

Cooperating Units: American Dental Association

Man Years:

Total:	2-1/4
Professional:	1
Other:	1-1/4

Project Description:

Objectives:

The objective of the present research project is to provide fundamental information relating to the regulation of catabolic processes in homofermentative streptococci and other groups of microorganisms. The immediate problems involve (1) a biochemically-oriented characterization of inducible enzymes responsible for the dissimilation of lactic and malic acid and (2) an understanding of the mechanisms employed by the microorganisms to control the synthesis and function of these enzymes.

Methods Employed:

All microbiological and biochemical procedures used in this study were adopted directly or derived from conventional techniques.

Major Findings:

1. The physiological conditions controlling the synthesis of a flavin-linked lactate oxidase system was the subject of last year's report. Since then, a preliminary characterization of the enzyme (s) responsible for the oxidation of lactate has

been made. The system is particulate in nature and is probably an integral part of the cell envelope. Attempts to solubilize the particle with detergents or surface-active agents have resulted in complete inactivation of the enzyme complex. However, the enzyme can be solubilized through extensive ultrasonic disruption. The flavin moiety was removed by chemical treatment and shown to be flavin mononucleotide; in the absence of the flavin cofactor the apoenzyme was rapidly inactivated.

2. The ability of homofermentative streptococci to grow at the expense of a variety of substrates including carbohydrates, polyalcohols, mono-and dicarboxylic acids is a well documented but unappreciated trait of this group of organisms. Many of the enzymes which catalyze the dissimilation of these organic substrate are apparently inducible and at the present time virtually nothing is known about the regulation of synthesis or function of this class of enzymes. One such enzyme has been studied this past year.

   The group D streptococci are capable of growing aerobically and anaerobically on L(+) malic acid. It was shown, that in the presence of malate, a "malic enzyme" is induced which converts the substrate to pyruvate and CO_2. The fate of pyruvate produced from malate differs radically from that produced during glucose catabolism. The latter is converted entirely to lactic acid while the former appears as acetate, ethanol and CO_2. This divergence in end products is a direct result of a requirement by lactate dehydrogenase for fructose-1, 6-diphosphate (FDP) which serves as an activator for the enzyme. Since FDP is not a direct intermediate product of malate catabolism, it does not accumulate in significant quantities and cannot activate the lactate dehydrogenase. Hence, pyruvate is diverted away from lactic acid towards acetate and ethanol.

   It has been demonstrated that the malic enzyme is not subject to catabolite repression by intermediate products of glycolysis and is synthesized during growth on glucose in a medium which also contains malate. However, despite the production and accumulation of malic enzyme in growing cultures containing glucose and malate, the latter was not utilized until the exogenous supply of glucose had disappeared. These observations prompted a thorough study of the factors influencing the regulation of enzyme activity.

   A procedure was developed which resulted in a 40-fold purification of the malic enzyme. The enzyme was subsequently characterized biochemically and physically. A specificity for malate as substrate and nicotine adenine dinocleotide (NAD)

as cofactor was demonstrated. Manganous and magnesium ion activate the enzyme; however, the former is far more effective. The enzyme has a molecular weight of between 85-95,000 and an optimal pH of 8.6. The equilibrium of the reaction is strongly in the direction of pyruvate and it is doubtful that the enzyme takes part in the biosynthesis of 4-carbon dicarboxylic acids.

A variety of compounds act as inhibitors of the purified enzyme. Oxaloacetate, a structural analogue of malate, is a competitive inhibitor. This was not unexpected and is a trait shared by all malic enzymes derived from a variety of sources. However, nucleotide triphosphates are also effective inhibitors as are 6-phosphogluconate (6-PG) and FDP. Through thermal inactivation and p-chloromercuriphenol sulfonic acid inactivation studies in direct evidence was obtained for the presence of inhibitor sites on the enzyme. Depending on the treatment, the enzyme could be desensitized to the respective inhibitors.

The roles played by the various inhibitors is not difficult to visualize. The inhibition by ATP and other nucleotide triphosphates provides a means for regulating the rate of malate dissimilation in a dynamic system. Any accumulation of ATP would cause a decrease in the rate of reaction, conversely, a decrease in the ATP pool would increase the reaction rate of the enzyme. The inhibition produced by FDP and 6PG would prevent malate dissimilation during aerobic or anaerobic glucose utilization.

A dissimilation of glucose and malate occurring simultaneously could easily produce an imbalance in growth by virtue of (1) a rapid accumulation of pyruvate which could produce a substrate inhibition of pyruvate-utilizing enzyme, or (2) by shifting the equilibrium between NAD and NADH far in the direction of reduced pyridine nucleotide. Either situation could potentially produce unbalanced growth and result in a grossly inefficient utilization of carbon and energy, or in cellular lysis.

Significance to Dental Research:

Knowledge of the physiology and regulation of metabolic processes in lactic acid bacteria is needed for better appreciation of the biological potentialities of oral streptococci and for understanding their behavior in the various environments where they are found. The present phase of the projects is of immediate significance for the problem of dental caries, since it attempts to define biochemical processes responsible for the production, accumulation, and subsequent utilization of lactic acid by homofermentative organisms.

Proposed Course of Project:

1. A further characterization of the lactate oxidase will be undertaken to elucidate the mode of catalysis. In addition, attempts will be made to demonstrate flavin-linked oxidative phosphorylation.

2. The study of the malic enzyme is near completion. However, some ancillary problems have arisen which require attention. Most pressing at this time is a clarification of the pathway by which energy is obtained and ethanol produced during malate fermentation. At present there is no known biochemical system which can catalyze such a reaction.

3. A variety of inducible enzymes will be studied on a comparative basis to learn whether regulation of this class of enzymes is achieved by a limited, select group of glycolytic intermediates, namely, fructose-6-phosphate and fructose-1, 6-diphosphate.

Part B:

Publications:

1. London, J. P., and Rittenberg, S. C.: Thiobacillus perometabolis nov. sp. A non-autotrophic thiobacillus. Arch. für Mikrobiol. 59: 218-225, 1967.

2. Smith, A., London, J. P., and Stanier, R. Y.: Biochemical basis of obligate autotrophy in blue-green algae and thiobacilli. J. Bacteriol. 94: 972-983, 1967.

3. Cohen-Bazire, G., and London, J. P.: Basal organelles of bacterial flagella. J. Bacteriol. 94: 458-465, 1967.

4. London, J.: Regulation and function of lactate oxidation in Streptococcus faecium. J. Bacteriol. 95: 1380-1387, 1968.

Serial No. NIDR-14 (65)
1. Microbiology
2. Immunology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Immunological Mechanisms in Oral and Systemic Disease

1. Significance of the Complement System to the Mechanism of Action of Endotoxin.

2. Studies on Biological Effectors of Immune Response and Immunological Tolerance.

Previous Serial Number: NIDR-9

Principal Investigator: Dr. S. E. Mergenhagen

Other Investigators: Dr. H. Gewurz, Dr. S. E. Berglund, Dr. R. Snyderman and Dr. R. J. Howard

Cooperating Units: Department of Microbiology, Temple University School of Medicine; Department of Microbiology, Johns Hopkins University School of Medicine; Department of Pediatrics and Microbiology, University of Minnesota Medical School; Max-Planck Institute for Immunobiology, Freiburg, Germany.

Man Years:

Total:	8-1/2
Professional:	5
Other:	3-1/2

Project Description:

Objectives:

1. To investigate immunological mechanisms by which oral and other microbial antigens incite tissue damage: mechanism of action of endotoxin through activation of the complement system.

2. To investigate the influence of viral and other biological effector systems on the immune mechanism.

Methods Employed:

Immunological analyses of bacterial and other antigens are carried out by immunoelectrophoresis, disc gel electrophoresis, Ouchterlony analysis, and passive hemagglutination techniques. Complement fixation and complement (C') component assays are performed with purified C' components in collaboration with Dr. Manfred Mayer and his colleagues at Johns Hopkins University. A modification of localized hemolysis in gel (Jerne technique) is employed for detection of antibody by lymphoid cells. Isotopically (I^{131} and I^{125}) labelled proteins are used in immune elimination studies. Column chromatography and sucrose gradient ultracentrifugation have been used for separation of serum antibodies and biologically-active polypeptides generated in serum with endotoxin. The Boyden chamber technique and the Schultz-Dale apparatus were used to study chemotaxis-generation and anaphylatoxin-generation *in vitro*.

Major Findings:

1. Interaction of the complement (C') system with endotoxic lipopolysaccharides (LPS). As previously reported (Bladen et al., J. Exp. Medicine, 125, 767, 1967), the C' system, by analogy with hemolytic C', exerts its maximum or nine-component effect upon interaction with LPS from gram negative bacteria. Unlike antigen-antibody precipitates, LPS incubated in fresh mammalian serum interacts most efficiently with the terminal components of C' (C'3, 5, 6, 7, 8, 9) with little fixation of C'1, 4, 2. This sequence of events is known to include as byproducts the promotion of phagocytosis and aggregation phenomenon, the generation of anaphylatoxin and production of factors chemotactic for neutrophils. It has long been known that endotoxin injection into the mammalian host induces aggregation of platelets and polymorphonuclear leukocytes, along with neutrophil chemotaxis and alterations of vascular permeability. During the past year we have performed experiments which support the hypothesis that endotoxin induced inflammation is mediated via the complement system.

   One such study sought to determine whether the generation of chemotactic factor by endotoxin in serum was dependent upon complement system activation. The Boyden chamber employing rabbit polymorphonuclear leukocytes *in vitro* was used for this study. Pre-heating serum, incubating at 0°C, or incubating in the presence of EDTA all prevented chemotactic factor generation as well as C' fixation by endotoxin. "Endotoxoids" deficient in C'- fixing activity were also deficient in chemotactic factor generation. Chemotactic

factor could not be generated by endotoxin sera of mice congenitally deficient in the C'5 component of complement while chemotactic factor was generated by endotoxin in the sera of coisogenic mice with normal C' levels for that species. The chemotactic factor induced by endotoxin was heat stable and non-dialyzable. Molecular sieve chromatography and sucrose density gradient ultracentrifugation demonstrated that the chemotactic factor was a relatively low molecular weight product (20,000-30,000) and as such differed from previously described C' system derived chemotactic factors. These experiments lend further support to the hypothesis that endotoxin-induced inflammation is dependent upon C' system activation. Furthermore, the relatively low molecular weight of this factor suggests that it might be derived from activation of a single complement component (perhaps C'5) rather than from complexing of multiple complement components.

In addition to our studies on chemotaxis, we have found that when endotoxin is added to normal undiluted guinea pig, rat or pig serum, a substance is generated with the characteristics of anaphylatoxin: i.e., it contracts guinea pig ileum and produces tachyphylaxis; its activity is blocked by antihistamines; and is not produced in heated serum or in the presence of EDTA, or at $0^{o}C$. Production of anaphylatoxin by endotoxin in guinea pig serum occurs with little consumption of C'1, 4, or 2, but correlates with consumption of C'3 and C'5. The relationship of the anaphylatoxin generating factor to the chemotactic factor is under investigation.

2. Immunoglobulin deficient sera and the role of antibody in endotoxin-complement interactions. Attempts to deplete antibodies to endotoxin by selective absorptions proved noncritical, in part because of solubilization of endotoxin. Therefore, endotoxin -C' interactions were investigated in certain agammaglobulinemic porcine, bovine and human sera. Endotoxins were reacted with pre-colostral piglet serum containing $< 2.5 \times 10^{-6}$mg % gamma globulin and with sow serum (500 mg % gamma globulins) derived from pathogen free Minnesota miniature pigs. Comparable C' fixation was observed in both groups of specimens. Over 80% of the piglet C' was fixed, neutrophil chemotaxis and anaphylatoxin were produced and characteristic C'- mediated lesions appeared on the endotoxin. Similar results were obtained with other immunoglobulin-deficient sera. The only human immune deficiency sera showing a lowered reactivity with endotoxin came from individuals with Swiss type lymphopenic agammaglobulinemia. However, unlike other human specimens, these sera were markedly deficient in the $C'1_q$ component

of C' and in "properdin". Further studies on the pathway for C' activation by endotoxin are under investigation.

3. Studies on effectors of the immune response.

(A) Antilymphocyte serum. Antilymphocyte serum (ALS) is a potent immunosuppressive agent. It has been found that mice readily form antibodies to the γG fraction of ALS. This prompted us to investigate whether a state of immunological tolerance to rabbit gamma globulin could alter the effectiveness of ALS. The prolongation of skin homografts by ALS is normal and tolerant mice was studied. Animals that were tolerant to rabbit gamma globulin, showed homograft survival times for longer than normal animals. These results have been attributed to a decreased rate of elimination of the active component of ALS in mice rendered tolerant to RGG.

(B) Influence of virus infection on the immune response. Our prior work showed that infection of mice with the lactic dehydrogenase virus (LDV) enhances the antibody response in mice and converts toleragenic doses of human gamma globulin into immunizing stimuli. During the past year a study was undertaken to determine the effect of LDV infection on cellular immune reactions by testing the ability of mice infected with LDV to reject skin homografts. In brief, median homograft survival times were significantly prolonged in mice infected with LDV. Thus, while LDV infection facilitates the production of humoral antibody and acts like an immunologic adjuvant, the same virus infection depresses cellular immunity. It is obvious that such studies have far reaching implications in tumor immunology and autoimmunity.

(C) Cellular and humoral antibody response to submucosally administered antigen in rabbits. Indirect evidence suggests that bacteria and their products which reside in diseased periodontal tissues stimulate an immune response. Our results have shown that small doses of bacterial antigens injected into the oral mucosa stimulate a marked immune response as indicated by increasing numbers of antibody-forming cells in regional lymph nodes and by increased serum antibody titers. Concomitantly, no detectable immune response occurs in the spleen or bone marrow. These studies suggest that low doses of bacterial antigens which gain access to periodontal tissues are adequate to stimulate an immune response which resides predominately in the regional lymph nodes and at local inflammatory sites.

Significance to Dental Research:

The role of immunological reactions in oral and systemic health and disease is a question of major concern to our understanding of disease pathogenesis. Studies concerned with interaction of microbial antigens and the immune mechanism of the host contribute to a better understanding of the inflammatory response and could clarify the role of bacterial products in oral and systemic disease. Studies of viral and other biological effectors of the immune response is important to an understanding of mechanisms of humoral and cellular immune reactions in the host.

Proposed Course of Project:

To continue our investigations as outlined above with particular emphasis on humoral and cellular mechanisms of host resistance.

Part B

Honors and Awards:

Dr. S. E. Mergenhagen: Recipient of the IADR Award for basic research in Oral Science, 1966. Drs. H. Gewurz, R. Snyderman, H. S. Shin, L. Lichstein, and S. E. Mergenhagen, paper entitled "Complement consumption by endotoxic lipopolysaccharide in immunoglobulin-deficient sera". Selected for presentation at the Plenary Session of the American Society for Clinical Investigations meeting held in Atlantic City, May 5, 6, 1968. Dr. H. Gewurz, invited participant at the Sannibel Research Conference on Developmental Immunology, Sannibel Island, Florida, February, 1968, paper entitled "Interactions of complement with endotoxic lipopolysaccharide".

Publications:

1. Bladen, H. A., Gewurz, H., and Mergenhagen, S. E.: Interactions of the complement system with the surface and endotoxic lipopolysaccharide of Veillonella alcalescens. J. Exp. Med., 125: 767-786, 1967.

2. Mergenhagen, S. E., Notkins, A. L., and Dougherty, S. F.: Adjuvanticity of lactic dehydrogenase virus: Influence of virus infection on the establishment of immunologic tolerance to a protein antigen in adult mice. J. Immunol. 99: 576-581, 1967.

3. Berglund, S. E., Markey, P. A., and Mergenhagen, S. E.: Observations on the kinetics of the hemolytic antibody response by localized hemolysis in gel over frozen sections of mouse spleen. Proc. Soc. Exp. Biol. Med., 126: 84-88, 1967.

4. Gewurz, H.: The immunologic role of complement. Hospital Practice, 2: 44-56, 1967.

5. Mergenhagen, S. E., Gewurz, H., Bladen, H. A., Nowotny, A., Kasai, N., and Luderitz, O.: Interactions of the complement system with endotoxins from a Salmonella minnesota mutant deficient in O-polysaccharide and heptose. J. Immunol., 100: 227-229, 1968.

6. Gewurz, H., Mergenhagen, S. E., Nowotny, A., and Phillips, J. K.: Interactions of the complement system with native and chemically modified endotoxins. J. Bacteriol. 95: 397-405, 1968.

7. Howard, R. J., Dougherty, S. F., and Mergenhagen, S. E.: Prolongation of skin homografts by rabbit anti-mouse lymphocyte serum in mice rendered tolerant to rabbit gamma globulin. J. Immunol., in press, 1968.

8. Gewurz, H., Pickering, R. J., Christian, C. L., Snyderman, R., Mergenhagen, S. E., and Good, R. A.: Decreased C'1$_q$ protein concentration and agglutinating activity in agammaglobulinemia syndromes: An inborn error involving the complement system. Clinical and Experimental Immunology, in press, 1968.

Serial No. NIDR-15 (67)
1. Microbiology
2. Virology
3. Bethesda, Md

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Recurrent and Persistent Viral Infections

Previous Serial Number: NIDR-10

Principal Investigator: Dr. A. L. Notkins

Other Investigators: Mr. W. K. Ashe
Dr. C. A. Daniels
Dr. S. E. Mergenhagen

Cooperating Units: National Cancer Institute

Man Years:

Total:	7-1/2
Professional:	3
Other:	4-1/2

Project Description:

Objectives:

This project concerns recurrent and persistent viral infections, the mechanism of virus sensitization and neutralization, the effect of virus infections on the immune system of the host, and the mechanism of action of lactic dehydrogenase virus (LDV).

Methods Employed:

The methodology was described in previous reports and new methods will be discussed under Major Findings.

Major Findings:

Sensitized Virus as an Indicator System for Studying Antiviral Immunoglobulins. Previous experiments showed that antiviral antibody could attach to herpes simplex virus (HSV) in the form of an infectious-virus antibody complex (sensitized virus) and that sensitized virus, in contrast to unsensitized virus, could be readily neutralized by anti-γ-globulin. Over the past year

we have studied a number of the factors and conditions involved in the neutralization of sensitized virus by anti-γ-globulin. Maximum neutralization of sensitized virus was found to depend upon: 1) the degree of sensitization; 2) the amount of free or unattached γ-globulin in the reaction mixture; 3) the concentration of anti-γ-globulin; 4) the nature of the sensitizing immunoglobulin; and 5) the specificity of the anti-γ-globulin. In addition, we found that the interaction between γ-globulin and anti-γ-globulin made it possible to use sensitized virus as a highly sensitive indicator system for studying antiviral immunoglobulins. Whereas the detection of a particular immunoglobulin by the immuno-diffusion method requires a sufficiently high concentration of antigen and antibody to produce a visible precipitate, a single sensitized virus particle can be detected as a result of replication and plaque formation. Neutralization of sensitized virus by specific anti-immunoglobulins thus makes it possible to detect and characterize the minute amount of antiviral immunoglobulin which is attached to the sensitized virion. In addition, we found the sensitized virus could be used to study and titer specific anti-immunoglobulins. Experiments in progress suggest that this technique might rival the hemagglutination system which is considered one of the most sensitive tools in immunology.

In addition, the anti-γ-globulin technique is proving to be an extremely useful serologic tool for demonstrating otherwise undetectable or low levels of antiviral antibody. For example, our experiments showed that the neutralization endpoint of certain antiviral sera was increased by as much as 64-fold when anti-γ-globulin was added to the reaction mixture. We hope that this technique will prove useful in the early serologic detection of certain viral infections.

Neutralization Inhibition Kinetics and Chronic Viral Infections. The anti-γ-globulin technique also has proved valuable in studying the kinetics of virus sensitization. For example, incubation of HSV with anti-HSV for 2.5 minutes resulted in little if any neutralization, but rendered 75% of the surviving virus neutralizable by anti-γ-globulin. The degree of sensitization increased with time and at 20 minutes over 99.8% of the surviving virus had become sensitized. Further experiments showed that sensitized virus was neutralized at a slower rate by antiviral antibody than unsensitized virus. The relationship between sensitization and inhibition of neutralization was studied by a technique which we refer to as neutralization inhibition kinetics. Basically, the procedure involves sensitizing the virus with different concentrations of anti-HSV and then determining the neutralization

rate constant of the sensitized virus by a second incubation with anti-HSV. Our data showed that as the degree of sensitization increased from 0 to 99.9%, the neutralization rate constant decreased from 14 for unsensitized virus to 1.8 for highly sensitized virus. This represents an 87% reduction in the rate of neutralization. The positive correlation between the degree of sensitization and the reduction in the neutralization rate constant supports the contention that the initial sensitization of the virus, sterically or otherwise, hinders the attachment of additional antiviral antibody and thereby retards or prevents further virus neutralization. Additional support for the argument that steric hinderance is responsible for the reduction in the neutralization rate constant comes from experiments in which we used papain digested antibody fragments. These experiments showed that sensitization of the virus with undigested anti-HSV γ-globulin reduced the neutralization rate constant by 53% when tested with undigested anti-HSV anti-globulin, but resulted in only 5% reduction in the neutralization rate constant when tested with the smaller univalent (Fab) antibody fragments. The demonstration by neutralization inhibition kinetics that sensitization with undigested anti-HSV inhibited the subsequent rate of neutralization by undigested anti-HSV but did not appreciably decrease the rate of neutralization by the smaller univalent Fab fragments points to the possibility that univalent antibody fragments might prove useful in neutralizing highly sensitized and otherwise resistant virus. The data from the HSV experiments and our previous findings with LDV also suggest that sensitization may play an important role in accounting for the chronic nature of certain virus infections.

Recurrent and Persistent Viral Infections of the Oral Cavity. Studies performed by Ashe and Rizzo on inapparent HSV infections in rabbits were published this year. In brief, they found that the higher the level of serum antibody the less susceptible was the oral mucosa to experimental infection with HSV. The severity of the oral lesions and the ability to culture virus from these lesions was found to be inversely related to the level of serum antibody. However, despite the presence of neutralizing antibody in the blood, infectious virus could at times be recovered from the saliva, even many months after the animals had been inoculated with the virus. In light of our recent findings with sensitized virus, it would be of interest to see whether the virus in the saliva is sensitized and if so whether it is more resistant to neutralization by antiviral antibody than unsensitized virus.

Over the past year, studies were continued on the biological and physical properties of the hemagglutinin associated with the rat submaxillary gland virus and the anti-hemagglutinin found in

the serum of rats. These experiments showed that the hemagglutinin was not found in the salivary glands of young rats but appeared at about 2 months and increased in titer with age. Further studies showed that an anti-hemagglutinin was present in the serum of young rats and that the titer of the anti-hemagglutinin increased with age and roughly paralleled the rise of the hemagglutinin. The data suggest that the early anti-hemagglutinin might be of maternal origin. Studies pertaining to the transmission of the rat submaxillary gland virus and the effect of surgical removal of the submaxillary gland on the titer of the hemagglutinin are in progress.

Effect of Viral Infections on the Immune System of the Host. One aspect of our studies on the effect of virus infections on the immune system of the host was brought to completion. We found that an acute virus infection (lactic dehydrogenase virus) could stimulate antibody production against a foreign protein (unaggregated human γ-globulin) that otherwise induces immunologic tolerance. In addition, our data suggest that one of the mechanisms of action of an immunologic adjuvant may be its ability to convert the tolerance-inducing components of a particular antigenic preparation into an immunogenic stimulus. Experiments in progress indicate that a virus infection also can effect the ability of the host to reject skin grafts. These and previously reported findings show that a virus infection can greatly influence the immune response of the host and points to the possibility that viruses also might play an important role in autoimmune diseases.

Significance to Dental Research:

A number of laboratories are now using the anti-γ-globulin technique for detecting sensitized virus, enhancing virus neutralization, and measuring low or otherwise undetectable levels of antiviral antibody. The use of sensitized virus as an indicator system for studying antiviral immunoglobulins should give new insight into the nature and properties of antiviral immunoglobulins in the saliva.

The development of the neutralization inhibition test has made it possible to study the effect of sensitization on virus neutralization. Our experiments showed that sensitized virus was neutralized at a slower rate than unsensitized virus. These findings suggest that sensitization might contribute to the chronic nature of certain virus infections and points to the possibility that herpes simplex virus might exist in saliva in the sensitized state.

Proposed Course of Project:

Present experiments are concerned with (1) the development and application of the anti-γ-globulin technique for the detection of sensitized virus; (2) the use of sensitized virus as an indicator system for detecting and characterizing antiviral immunoglobulins; (3) the extension of the neutralization inhibition test to other viruses and classes of immunoglobulins; (4) the effect of virus infections on the immune system of the host; and (5) the detection and characterization of antiviral antibody in human saliva and attempts to recover sensitized virus from the oral cavity.

Part B Publications:

1. Ashe, W. K., and Rizzo, A. A.: Inapparent herpes simplex virus infection in inoculated rabbits. Proc. Soc. Exptl. Biol. Med., 124: 1150-1154, 1967.

2. Rizzo, A. A., and Ashe, W. K.: The influence of different levels of serum antibody on the susceptibility of rabbit oral mucosa to experimental herpes simplex virus infection, Arch. Oral Biol., 12: 933-936, 1967.

3. Mergenhagen, S. E., Notkins, A. L., and Dougherty, S. F.: Adjuvanticity of lactic dehydrogenase virus: Influence of virus infection on the establishment of immunologic tolerance to a protein antigen in adult mice, J. Immunol., 99: 576-581, 1967.

4. Ashe, W. K. and Notkins, A. L.: Kinetics of sensitization of herpes simplex virus and its relationship to the reduction in the neutralization rate constant, Virology, 33: 613-617, 1967.

5. Notkins, A. L., Mage, M., Ashe, W. K., and Mahar, S.: Neutralization of sensitized lactic dehydrogenase virus by anti-γ-globulin. J. Immunol., 100: 314-320, 1968.

6. Hampar, B., Notkins, A. L., Mage, M., and Keehn, M. A.: Heterogeneity in the properties of 7S and 19S rabbit neutralizing antibodies to herpes simplex virus. J. Immunol., 100: 586-593, 1968.

Serial No. NIDR-16 (64)
1. Microbiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: The Mechanisms by which Bacterial Products May Cause Destruction in Human Periodontal Disease

Previous Serial Number: NIDR-11

Principal Investigator: Dr. A. A. Rizzo

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	2-1/4
Professional:	1
Other:	1-1/4

Project Description:

Objectives:

1. To investigate the diffusibility of endotoxins and antigens into rabbit gingival pocket tissues.

2. To investigate the in vivo toxicity of bacterial products to rabbit corneal and gingival tissues under conditions simulating those of the human mouth.

3. To determine whether hydrogen sulfide production in human periodontal pockets is related to periodontal disease.

Methods Employed:

Much of the methodology used in these studies has been described in previous annual reports (NIDR-12, 1967 ; NIDR-12, 1966). One method used this year, but not previously employed, included the use of a vascular labelling technique to show that topically applied bacterial agents such as hydrogen sulfide can cause an increase in vascular permeability in oral tissue. Another modification applied this year was to use as a test tissue for

topical toxicity experiments an area of lip mucosa which resembles gingival sulcus tissue, but which can be irrigated without any mechanical trauma.

Major Findings:

1. Gingival absorption of endotoxins and antigens.
Using topical administration by means of locally placed cotton wicks containing endotoxins and antigens, rabbit ingival pocket tissues were tested for absorption of these agents. Such tests, reported in a previous annual report (NIDR-12, 1967), indicated that these high molecular weight substances would not penetrate an intact epithelial sulcular lining. This year these results have been confirmed and extended by using prolonged irrigation of the gingival tissues with high concentrations of endotoxins and antigens. When the pocket lining was ulcerated, prolonged irrigation with concentrations of endotoxin as low as 0.01 μg/ml resulted in sufficient penetration to evoke an antibody response in regional lymph nodes.

2. In vivo toxicity of bacterial products.

   a. Electron microscopy of rabbit corneas irrigated with neutral solution of ammonia has elucidated the nature of the toxic effect of ammonia on the anterior epithelium of this organ. The principal morphologic alterations were the presence of intracytoplasmic vacuoles up to 10-15 microns in diameter and nuclear shrinkage. There was no apparent disruption of cytoplasmic, nuclear, or basement membranes, nor of desmosomes, and no alteration in the underlying structures of the stroma.

   b. Microscopic studies of corneas irrigated with neutral solutions of hydrogen sulfide have revealed changes throughout the epithelium. Surface cells were observed to degenerate and become stripped off, and basal nuclei showed obvious swelling. Superficial cells had lost their cytoplasm, and the nuclei had undergone swelling before becoming exfoliated. The process of degeneration and detachment resulted in formation of a shallow crater in the surface outline and a thinner integument.

   c. Findings not mentioned previously in relation to sulfide irrigation of eyes included evidence that the gas might be diffusing through the cornea into the aqueous

humor and iridial tissues. Sulfide treated eyes showed slower and diminished pupillary constriction to light compared to controls, and the iridial vasculature exhibited an increase in permeability when tested by the vascular labelling method. Specific chemical tests indicated that hydrogen sulfide appeared in the aqueous humor very soon after irrigation with relatively low concentrations of sulfide had begun.

d. Irrigation of rabbit gingiva and lip mucosa with hydrogen sulfide produced gross edema, and erythema which was quite dramatic at higher concentrations, but was still evident at concentrations as low as 0.01 molar. Increased vascular permeability was demonstrated in such specimens by means of the vascular labelling technique. Preliminary histologic examination of hydrogen sulfide-treated gingival specimens showed epithelial changes similar to those observed in cornea. In addition, widened intercellular spaces were observed in both deep and superficial areas of the lining epithelium.

Significance to Dental Research:

A serious obstacle to disease-oriented research in the periodontal field has been the lack of suitable test systems to study directly the effects upon tissues of the many substances chronically in contact with the human gingival tissues. The methods which have been developed and applied in the present studies have provided meaningful information on *in vivo* local toxicity of ammonia and hydrogen sulfide under conditions simulating those of the human mouth. Since both of these substances are known to be produced by bacteria in the human mouth, the demonstration that low levels of these agents can produce epithelial damage in a neutral, isotonic milieu may be of considerable importance in the initiation of periodontal disease. It is of possible greater significance that short-term exposure to neutral solutions of hydrogen sulfide not only causes epithelial alterations, but also induces gingival edema and erythema, two well known clinical signs of human periodontal disease.

Tests on the absorption of an antigenic protein and of bacterial endotoxin indicate that these agents can diffuse into gingival tissues and cause alterations only after ulceration has taken place. Thus, the role of these non-enzymic, high molecular weight substances may be more important in the progression, rather than in the initiation of periodontal disease.

Proposed Course of Project:

1. To define further the pathologic alterations induced by ammonia and hydrogen sulfide in experimental systems. These studies are to include light and electron microscopic studies of eye and oral specimens exposed to ammonia, hydrogen sulfide, and to mixtures of these agents.

2. To compare the pathologic alterations in inflammed human gingival tissues to the alterations induced by ammonia and hydrogen sulfide in experimental tissues, using criteria developed in the proposed studies described above (Item 1).

3. (a) To carry out additional studies on hydrogen sulfide production in human periodontal pockets to determine whether or not the production of this gas shows a clear-cut association with periodontal disease.

   (b) To estimate the concentrations of hydrogen sulfide which actually develop in healthy and diseased periodontal sites in humans.

4. To test ammonia and hydrogen sulfide as vehicles for aiding in the transmission into gingival tissues of high molecular weight bacterial products such as antigens and endotoxins and of bacterial cells themselves.

Part B:

Publications:

1. Rizzo, A. A.: The possible role of hydrogen sulfide in human periodontal disease. I. Hydrogen sulfide production in periodontal pockets. Periodontics 5: 233-236, 1967.

2. Rizzo, A. A.: Rabbit corneal irrigation as a model system for studies on the relative toxicity of bacterial products implicated in periodontal disease. The toxicity of neutralized ammonia solutions. J. Periodontol., 38: 491-499, 1967.

3. Rizzo, A. A.: Absorption of bacterial endotoxin into rabbit gingival pocket tissue. Periodontics, 6: 65-70, 1968.

4. Rizzo, A. A.: Summary of papers on biochemistry, physiology and microbiology of the periodontium, in Proceedings of the International Symposium on Oral Diseases at the University of Alabama, 1968. Ala. J. Med. Sci. In press.

Serial No. NIDR-17 (67)
1. Microbiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Systematic Microbiological Taxonomic Studies

Previous Serial Number: NIDR-13

Principal Investigator: Mr. Morrison Rogosa

Other Investigators: Dr. M. I. Krichevsky
Dr. J. P. London

Cooperating Units: Division of Computer Research and Technology, N.I.H.
University of Maryland
American Type Culture Collection
Georgetown University
Bergey's Manual Trust
International Subcommittee on Lactobacilli and Related Organisms
International Subcommittee on the Bifid Bacteria

Man Years:

Total:	2-1/4
Professional:	1
Other:	1-1/4

Project Description:

Objectives:

To determine the systematic taxonomic relationships of microbial organisms, with particular reference to members of the oral microbiota.

Methods Employed:

Sophisticated modern techniques are applied in the study of morphological, culture, biochemical, genetic, immunological, ecological, and pathogenic characteristics of microorganisms. Computer technology is employed to store, retrieve, and assess the significance of data obtained in this laboratory, as well as information from other world laboratories, in the synthesis of a comprehensive systematic microbiological taxonomy of global usefulness.

Major Findings:

The anaerobic gram negative organisms, whose nutritional characteristics were described previously were found to be amino acid fermenters, particularly of glutamic acid, with the production of 2 moles acetic/1 mole butyric acid, plus CO_2. A relatively simple gas chromatographic method, developed in this laboratory, was employed for the quantitiative detection of the lower fatty acid homologues. Organisms previously studied by others as Micrococcus aerogenes appear biochemically similar to our isolates. Electron micrographs indicate both groups of organisms have characteristic gram negative structure. Accordingly, they appear to be members of the genus Peptococcus. Thus through our work, the delineation of Peptococcus has been now achieved.

The studies on the utilization of malate and citrate by Lactobacillus casei and Lactobacillus Plantarum are continuing. The studies of cell wall and somatic antigens in lactobacilli, in cooperation with C. A. Mills and P. A. Hansen of the American Type Culture Collection, have shown that agglutinating antigens are present in cell wall and somatic teichoic acids, and that group precipitating antigens may be associated with the cell wall teichoic acids only. A manuscript describing these findings is being prepared.

As Chairman of the Bergey's Manual Committee on non-spore-forming gram-positive rods, the principal investigator conducted a meeting of the European members in London, England on March 1-3, 1968 inclusive. Twenty bacterial genera, including a number of oral importance, were discussed and plans made for their improved definition in the forthcoming edition of Bergey's Manual. This meeting was mandatory. Further European meetings, one to be held this summer in England, are indispensable for the successful conduct of the work.

A comprehensive questionnaire, to be answered by world specialists in various genera, was prepared in this laboratory with the cooperation of Dr. Krichevsky and Dr. Colwell of Georgetown University. Dr. Colwell is an acknowledged world expert on the use of computers in bacterial taxonomy. This questionnaire is being submitted to the world scientific community for consideration. The questionnaire is designed so that answers can be computer coded, making it possible for the data to be stored, retrieved and assessed with computer aid. Through this program the National Institutes of Health has a unique opportunity to render an invaluable service to world academic science and clinical diagnosis.

Significance to Dental Research:

Many problems exist in the characterization of indigenous oral microorganisms and their natural relationships with other organisms. Systematic taxonomic studies are indispensable for the advancement of academic and clinical science.

Proposed Course of Project:

1. Utilization of computer technology to store, retrieve and analyze data of characteristics of 265 genera of bacteria, in cooperation with Drs. Krichevsky and Colwell, the Bergey's Manual Trust, and the International Committees concerned with the definition of various genera.

2. Studies of the genetic relatedness of lactobacilli, and their macromolecular biology; continued analysis of their antigenic relatedness; studies of their malate and citrate metabolism; investigations of their potential aerobic or nonglycolytic metabolism.

3. Continued studies of utilization of lactate and other metabolizable substrates by Veillonella species and related organisms; studies of gluconeogenesis in Veillonella species.

Part B

Honors:

Member of the Subcommittees on lactobacilli and related organisms; Neisseriaceae; and Bifidobacterium of the International Committee on Bacteriological Nomenclature. Author of Research Proposal accepted by the Subcommittee on Bifidobacterium.

Member of the merican Society for Microbiology Committee on Lactobacilleae and Propionibacteriaceae.

Appointed Chairman of the Bergey's Manual Committee on Gram-Positive Non-Sporulating Rods.

Appointed by the Bergey's Manual Committee on Gram-Negative Anaerobic Bacteria to rewrite the description of the genus Veillonella.

Appointed to the Board of Editors of Bacteriological Reviews.

Publications:

Rogosa, M. and L. L. Love: Direct quantitative gas chromatographic separation of C_2-C_6 fatty acids, methanol, and ethanol in aqueous microbial fermentation media. Applied Microbiology, 16: 285-290, 1968.

Serial No. NIDR-18 (61)
1. Microbiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Studies on the Regulation of Lactic Acid Production by Microorganisms.

Previous Serial Number: NIDR-14

Principal Investigator: Dr. C. L. Wittenberger

Other Investigators: None

Cooperating Units: American Dental Association

Man Years:

Total:	3-1/4
Professional:	1
Other:	2-1/4

Project Description:

Objectives:

It is the general purpose of this program to examine mechanisms by which the biochemical activities of the microbial cell are regulated, and to delineate, where possible, the molecular basis for such regulation. The specific biochemical process currently under investigation is that of anaerobic lactic acid metabolism. One organism employed in these studies, Butyribacterium rettgeri, can ferment glucose with the formation of lactic acid or, under appropriate conditions, it can utilize lactate as a substrate for growth. This single organism, therefore, provides an excellent model system for studying various biochemical aspects of both lactate formation and lactate degradation. In addition, studies have been initiated to ascertain what factors may operate to regulate the formation of lactic acid in members of the genus Streptococcus.

Methods Employed:

All are standard techniques routine to the type of studies herein described.

Major Findings:

Prior studies relating to the regulation of lactic acid metabolism in Butyribacterium rettgeri dealt with a detailed analysis of certain factors which influence the catalytic activity of the enzyme (lactate dehydrogenase) involved in the conversion of pyruvic acid to lactic acid. It was established that this enzyme possessed two binding sites for its substrate, pyruvate, and a site for binding adenosine triphosphate (ATP) which was separate and distinct from any occupied by the other ligands involved in the reaction. Interaction of the enzyme with ATP resulted in an inhibition of its catalytic function. This inhibition of the conversion of pyruvate to lactate by ATP provides the cell with a means by which it may free pyruvate carbon for utilization in biosynthetic pathways when sufficient energy (ATP) is available for such endergonic processes.

One phase of our present investigation has been oriented toward resolving the mechanism by which ATP inhibits the B. rettgeri lactate dehydrogenase (LDH). This is part of a general effort to understand, at the molecular level, those processes which operate within the cell to regulate its biochemical activities. The results from these studies have led to the formulation of a model for the LDH which appears to explain satisfactorily its various physical and kinetic properties.

The proposed model predicts that interactions of the enzyme with ATP or NADH result in a partial restriction at the site for the unbound ligand. That such ligand-protein interactions do indeed result in conformational alterations of the protein is indicated by the fact that ATP confers a marked thermal stability to the enzyme while NADH reverses the protective effect of ATP. The configurational changes which accompany binding of the coenzyme or nucleotide inhibitor, appear to involve alterations in the tertiary structure of the protein rather than alterations of its quaternary structure. For example, no significant change in the molecular weight of the LDH can be detected by either gel filtration or sucrose density gradient centrifugation as a result of its interaction with either of the ligands.

Lactic acid formation has also been studied in members of the genus Streptococcus. In S. faecium, the LDH requires fructose-1, 6-diphosphate (FDP) for activation. The enzyme is virtually completely inactive in the absence of FDP and the requirement for FDP as the activating ligand is highly specific. A variety of metabolic intermediate compounds have been tested for their ability to activate the LDH without success.

The LDH has been purified about 100-fold over the present in cell-free extracts of S. faecium and this purified enzyme has been used to study the mechanism of FDP activation. Preliminary results suggest that FDP activates the enzyme by mediating a conformational alteration of the LDH which makes both the coenzyme (NADH)- and substrate (pyruvate)-binding sites more accessible to the respective reactants. It has been shown, for example, that FDP lowers the apparent K_M for both NADH and pyruvate.

Finally, it has long been known that the streptococci produce more lactate from glucose at an acid pH than at an alkaline pH. We have found that the S. faecium LDH binds its activator (FDP) very well at an acid pH but very poorly at an alkaline pH. This probably provides a mechanistic explanation for the established physiological relationship between pH and lactic acid production.

Significance to Dental Research:

It is anticipated that information derived from these studies on biochemical control mechanisms will have general significance in advancing the knowledge of cell physiology. Such knowledge is of fundamental importance in formulating rigorous and systematic approaches to such diverse problems as cell reproduction, chemotherapy, and viral alteration of host metabolism. Present work on the regulation of microbial lactic acid production should further contribute specifically to a more comprehensive understanding of the factors involved in dental caries.

Proposed Course of the Project:

Present studies on the regulation of lactic acid production in streptococci will be continued. Special emphasis will be given to establishing the precise mechanism by which the lactate dehydrogenase from these organisms is activated by fructose-1, 6-diphosphate. Other studies with the streptococci will also be initiated to determine whether intermediate compounds from one catabolic pathway can influence other pathways of catabolism.

Part B: Publications

1. Wittenberger, C. L.: Kinetic studies on the inhibition of a D(-)-specific lactate dehydrogenase by adenosine triphosphate. J. Biol. Chem., 1968, In press.

Report of the Laboratory of Biochemistry
National Institute of Dental Research
Summary Statement

With the creation of the Connective Tissue Section, the appointment of a Chief for the Enzyme Chemistry Section, and the relocation (from Human Genetics Branch) and renaming of the Cell Biology Section, all laboratory personnel are now assigned to one of five sections which reflect the major programs of the laboratory. Since the sections are all located on one floor, there is considerable interchange and collaboration.

These administrative and program changes mark the close of all studies on fluoride metabolism and experimental caries in this laboratory with the exception of one study on the long-term effects on bone and aorta of water floridation in Grand Rapids. This study is being continued under contract and should be completed by the end of 1968. The present efforts of the laboratory represent broadly based and fundamental biochemical programs designed to have relevance to the modern concept of dental research.

In all of its programs, the laboratory continues to depend upon approximately equal proportions of senior research staff and postdoctoral personnel here under one of the several NIH programs or as guest workers. Postdoctoral training is, therefore, an important byproduct of the laboratory operation as well as an important source of scientific talent.

For purposes of summarization, the program of the laboratory is divided according to the section designations.

Protein Chemistry Section

The major effort for some years has been the study of the structure of collagen in an attempt to understand the factors involved in stabilization of molecular structure, fibril formation (aggregation) and maturation (covalent cross-linking). The large size of the molecule requires a stepwise "taking apart" of the molecule to smaller pieces amenable to chemical and physical chemical studies. The three α chains can be isolated chromatographically and the α chains can be cleaved chemically at specific positions (methionyl residues) with CNBr to yield a relatively simple mixture of unique peptides. In addition to the $\alpha 1$ chain of rat skin collagen which has been studied in this way, the $\alpha 2$ chain of rat skin collagen and the $\alpha 1$ and $\alpha 2$ chains of chick bone collagen are now being investigated. The results of these studies plus results on other collagens being studied elsewhere permit some important conclusions. First, collagens from different species differ but there is close homology. Comparisons show what portions of the amino acid sequence are critical and provide a basis for relating structure to function. Second, collagens from different tissues of one species may have identical sequences. For example, the nonhelical cross-link region of the $\alpha 1$ chain, which presumably has a critical role in maturation and aggregation, is identical in chick bone and skin collagen and in rat skin and tail tendon collagen but the rat and chick collagens differ from each other in one residue of the

nineteen in the region. Human skin collagen differs in several positions. The apparent identity of the primary structure of the two collagens from rat and of the two collagens from chick suggests that collagens in different tissues of one animal may arise from a single gene (or group of genes). Differences in function must then be ascribed to differences between the collagens introduced after polypeptide chain assembly (such as the degree of hydroxylation and of aldehyde formation) or to other tissue components.

The chemistry of the cross-links in collagen is under further investigation. Chick bone collagen has been shown by chemical and x-ray diffraction studies to have a high degree of intermolecular cross-linking. Preliminary results suggests it is a suitable tissue for study to determine the sites and mode of intermolecular cross-linking.

The amino acid sequence of 54 residues at the amino terminal end of the $\alpha 1$ chain of rat skin and rat tail tendon collagen are now known. Studies in progress here and in laboratories outside NIH are increasing this number with the hope that eventually the entire sequence of about 1000 amino acids can be determined.

The availability of peptides from collagen of known sequence provides an usual opportunity to study collagen helix formation in a well-defined system. Studies in progress show that a random coil peptide can convert in a completely reversible reaction to the collagen helix by aggregation to a trimer producing a triple-chain helix. The kinetic and thermodynamic parameters of the process can be calculated.

The same process of reversible helix formation has been utilized under other conditions in a mechanochemical study. Solutions of certain salts denature or shrink (loss of helix) collagen fibers. If the fibers are stretched under force, helix is regained. It was shown that interaction with the salt is greater in the shrunken than in the stretched state. A theoretical model was derived for the process and shown to be consistent with the experimental data.

Connective Tissue Section

Current studies are largely in two areas--cross-linking of collagen and elastin and mineralization of connective tissue. Previous studies established that the initial step in the cross-linking of collagen and elastin is identical. Lysine in peptide linkage is converted to α-amino adipic- -semialdehyde (allysine). A major breakthrough was made with the finding of an enzyme in extracts of connective tissue that carries out this reaction. The enzyme is inhibited *in vitro* by levels of a lathyrogen which previously have been shown to block collagen and elastin cross-linking *in vivo* and in culture. Penicillamine, another compound inhibiting the cross-linking of collagen and elastin, has been found to block the cross-linking of elastin after the formation of allysine. In the presence of penicillamine an altered elastin, rich in allysine but deficient in cross-links, accumulates.

Elastin from aorta and nuchal ligament was used in an *in vitro* system to study mineralization. The amino acids located at the nucleating site of

mineralizing elastin were isolated by using elastase to remove nonmineralized matrix. The mineralized organic residue following digestion was found to be rich in cysteine and dicarboxylic amino acids. Fe^{+2} was found to be concentrated at this site. Model studies showed that a ternary complex of Fe^{+2}, phosphate and complexing agent is the nucleating species. Presumably, in elastin, Fe^{+2} reacts with cysteine, and phosphate fills the unoccupied coordination positions of this complex. This local concentration of oriented phosphates acts as the seed upon which calcium and other phosphates ions are deposited.

A second system to study mineralization is provided by embryonic chick bone. This tissue mineralizes when maintained under proper conditions in vitro. Bones obtained from animals just prior to mineralization require embryo extract for mineralization and to promote a high growth rate and differentiation. However, bones obtained after mineralization has started will continue to mineralize in the absence of embryo extract in a manner that is largely independent of growth or further differentiation. In the young, nonmineralized bones, only two cell types (cartilage and fibroblasts), can be obtained in cell culture after trypsinization. A third type of cell is obtained from the shaft of mineralizing bones. This cell, appearing at the site and time of mineral deposition, apparently initiates this process.

Enzyme Chemistry Section

Studies carried out in this section have been primarily focused on the basic mechanisms by which enzymes function as organic catalysts. Two enzymes that catalyze very different biological reactions, porcine pancreatic chymotrypsin C and guinea pig liver transglutaminase, have been under intensive study. A sequence of twelve amino acids surrounding the essential histidine of the chymotrypsin C active site has been elucidated. Certain differences between this sequence and those in other chymotrypsins of other mammals may reflect species differences and determine specificity. A kinetic mechanism for transglutaminase action, consistent with all of the experimental observations, has been formulated. This mechanism, wherein a common acyl enzyme intermediate is formed, takes into consideration the metal ion activation of the enzyme and explains the varied calcium ion concentration dependency for the several reactions catalyzed by transglutaminase. There appear to be two separate metalbinding sites and a conformational change accompanies the binding.

Pharmacology Section

The emphasis of recent studies has been on drug-induced fetal malformations with a particular interest in cleft palate. Normal mammalian palatogenesis consists of horizontal rotation of the platal shelves and subsequent fusion. Prior to and during the process there is a gradual reduction in the flexure of the cranial base. It is proposed that this straightening results in an "internal shelf force" which raises and rotates the palatal shelves.

Failure to effect closure could result if the shelves do not rotate or if fusion fails even though rotation is normal. The process of fusion was studied by histochemical techniques. Enzyme changes associated with epithelial breakdown at the point of fusion were observed.

In view of the complexity of the process, it is not surprising that palatogenesis can be effected by many routes. A new example was provided by the observation that lathyrogens, which have as their primary effect the inhibition of cross-linking of collagen and elastin, can produce cleft palate.

A new approach to the study of teratogens was developed. Techniques were devised whereby a drug or control compound in a millipore filter can be applied directly to the embryo reducing the contribution of maternal and placental factors.

The study of teratogenesis in the primate has been conducted under contract. Thalidomide, a known teratogen, was used as a positive control and produced malformations in the Rhesus monkey. If given at the appropriate time, very low doses were active in producing a variety of malformations.

Cell Biology Section

Current investigations are aimed at an understanding of the mechanisms bywhich genetic information contained within the cell functions in the regulation of normal and abnormal cell growth and cellular differentiation. The major effort consists of a multidirectional approach to the question of lymphocyte growth. In this unique system, normal human cells can be studied in an essentially physiological resting state in vitro, and then are induced to enter a state of rapid growth and division by contact with a variety of stimulating agents, particularly phytohemagglutinin (PHA) from kidney beans.

As background to detailed studies, quantitative and kinetic data have been obtained, determining the optimal dose of growth-stimulating agents, the time course of the growth response, and the interactions between cell growth and cell death which occur at excessive doses of stimulating agent. Current evidence suggests that the same mechanism which stimulates the lymphocyte to grow may, when carried to extremes, kill the stimulated cell.

Studies with polycations (DEAE-dextrans) and polyanions (heparin, dextran sulfate) suggest that an important event in initiating and permitting continuation of cell growth occurs at the cell surface, and have further shown that the surfaces of malignant lymphoid cells differ from those of normal lymphocytes in their reactivity with polyions. Some growth stimulating agents, particularly specific antigens, were shown to require an interaction between lymphocytes and macrophages in order to produce effective growth stimulation. This finding is consistent with the widely held notion that macrophages must process antigens in order to make them recognizable by the lymphocyte.

Immediately after stimulation of lymphocytes by PHA, RNA synthesis increases. At least three steps which govern the production of various classes of RNA have been identified. Controls at each step are altered after growth stimulation directing a specific pattern of changes which characterize the shift of resting cells to a growing state. A large increase in the rate of ribosomal RNA synthesis accounts in part for the progressive cytoplasmic accumulation of ribosomes, increased protein synthesis, and cellular enlargement found in growing cells.

The evidence indicates that the non-growing lymphocyte contains a limiting amount of protein (or proteins) required for ribosomal RNA synthesis. It further suggests that this protein must be constantly produced to allow the increased ribosomal RNA synthesis that follows growth stimulation. The availability of this protein may therefore constitute a control mechanism in governing lymphocyte growth.

The pre-existing ribosomes of the resting lymphocyte were found to be capable of increased protein synthetic activity, implying that the availability of ribosomes is not a limiting factor in determining cell protein synthesis in the resting state. The resting cell thus has the capacity to increase the production of proteins needed for ribosome and ribosomal RNA synthesis without any need for new ribosomes. However, one new ribosomes are available, the synthesis of the various products needed for multiplication can proceed.

In separate experiments, a hitherto unreported double-stranded RNA form has been found in animal cells by the use of new analytical techniques. This material was found in small amounts in lymphocytes, but in much larger quantity in malignant lymphoma cells.

Serial No. NIDR-19 (52)
1. Biochemistry
2. Protein Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Analytical and Structural Studies on Collagen

Previous Serial Number: NIDR-30

Principal Investigator: Dr. K. A. Piez

Other Investigators: Dr. P. Fietzek, Dr. J. Daniels, Dr. E. Schiffmann and Dr. M. Rubin

Cooperating Units: Dr. P. Bornstein (Univ. of Washington, Seattle), Dr. W. Butler (Univ. of Alabama, Birmingham), Dr. A. Kang (Massachusetts General Hospital, Boston)

Man Years:

Total:	5 1/2
Professional:	4
Other:	1 1/2

Project Description:

Objectives:

It is the long range purpose of this project to study the chemistry and structure of collagen as it relates to function. The immediate problems are: (1) characterization of the α chains of collagen, including limited amino acid sequencing; (2) the chemistry and biosynthesis of covalent cross-links in collagen; and (3) the renaturation of collagen polypeptide chains into the collagen triple-helical structure.

Methods Employed:

Usual laboratory techniques.

Major Findings:

The characterization of the α chains of collagen has been approached by utilizing a specific reagent for cleavage (CNBr). Eight peptides accounting for the entire α1 chain have been isolated. The order of these peptides has now been determined. This information is necessary to assemble amino acid sequences of the individual peptides as they become available.

110

Amino acid analyses and partial sequences are becoming available for several of the peptides from several different collagens. The sequence around the cross-link region of α1 is identical in chick skin and chick bone collagens and in rat skin and tail tendon collagens. The chick and rat collagens differ, but only in a single amino residue in a total of 10 residues. This pattern holds for other peptides suggesting that different connective tissue collagens in the same animal are derived from the same gene. If this finding is confirmed as a more complete comparison is made, tissue differences must be ascribed to differences in the collagen that are introduced after polypeptide synthesis (such as in the degree of hydroxylation or of aldehyde formation prior to cross-linking) or to other tissue components.

Specific cleavage of the α2 chain of rat skin collagen with CNBr produces five or six unique peptides. These have been isolated and partially characterized.

The CNBr peptide (α1-CB6) from the carboxyl terminal end of α1 from rat skin collagen, which has a molecular weight of about 16,000, is being studied to see if it has any unusual features analagous, or complementary, to the amino terminal end where cross-links originate. Cleavage of α1-CB6 with chymotrypsin yields three peptides which have been isolated and will be characterized.

The chemistry of the intramolecular cross-link in rat skin collagen is being pursued. Indirect evidence has suggested that two lysine-derived aldehydes (allysine) condense to form an aldol product. Assuming this to be correct, a series of organic reactions has been devised to degrade the cross-link and yield specific products which will prove the structure. Preliminary results are consistent with the proposed structure.

The nature of the forces that hold the three chains of collagen together in a triple chain helix is not well understood. This problem has been approached by using a small peptide (36 amino acid residues) to study helix formation and denaturation. It has been found that completely reversible helix formation occurs and the kinetic and thermodynamic parameters of the process can be calculated. This simple system serves as a model for the more complex α chains which have about 1000 residues and form a perfect triple helix *in vivo*.

The collagen fiber has been used as a model to study the effect of environment on conformation. In the presence of certain aqueous salt solutions the shrinkage temperature (a measure of loss of conformation) of a collagen fiber is markedly lowered. This phenomenon is an example of a mechanochemical process. Although

this particular process is not utilized by living organisms to do work, it is analogous to living processes and has the advantage of being amenable to study. The parameters of work (measured as stretching force or tension on the fiber), conformation (measured as length of the fiber) and salt (LiBr) concentration were related in a theoretical model. Experimental data were obtained consistent with the model. It was found that the interaction of salt and collagen was force and conformation dependent.

Part B Publications:

Bornstein, P.: The incomplete hydroxylation of individual prolyl residues in collage. J. Biol. Chem., 242:2572-2574, May 1967.

Piez, K. A.: Soluble collagen and the components resulting from it denaturation. In Ramachandran, G. N. (Ed.): Treatise on Collagen, Vol. I, Chemistry of collagen. Academic Press, 1967, pp 207-252.

Piez, K. A., Bornstein, P., and Kang, A. H.: The chemistry and biosynthesis of interchain cross-links in elastin. In Fibrous Proteins. Butterworths, in press.

Bornstein, P.: Comparative sequence studies of rat skin and tendon collagen. I. Evidence for incomplete hydroxylation of individual prolyl residues in the normal proteins. Biochemistry 6, 3082-3093, Oct. 1967.

Piez, K. A.: Molecular weight determination of random coil peptides from collagen by molecular sieve chromatography. Anal. Biochem., in press.

Serial No. NIDR-20 (66)

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Factors Influencing Resorption and Cell Growth in Collagen Implants Formed by Thermal Gelation *in vivo*

Previous Serial Number: NIDR-32

Principal Investigator: Dr. K. A. Piez

Cooperating Units: None

Man Years: None

Project Description:

Objectives:

This project was established under contract as an extension of intramural metabolic and chemical studies on the maturation and chemistry of collagen. The immediate objective is to obtain information concerning the fate of collagen reconstituted *in vivo* with particular interest in its resorption and maturation through cross-linking. A longer term objective is to determine the potential of reconstituted collagen as a filling material for excisional wounds in hard and soft tissue.

Methods Employed:

This project is conducted largely by Dr. S. Shoshan, Hadassah School of Dental Medicine, Jerusalem, Israel, under contract utilizing PL 480 funds. (Research Agreement No. 645141 in the amount of 249,741 Israeli Pounds for the period December 15, 1965 to December 15, 1968.) The project employs half the time of Dr. Shoshan and 2-1/2 man years by supporting personnel.

The project first requires an *in vitro* study of the conditions under which purified collagen can be reconstituted to native type fibrils. Using the established conditions, collagen is reconstituted *in vivo* in experimental animals in such a way that the implant can be removed at a later time and examined by histological and chemical methods. Studies during the current year utilized diffussion chambers containing purified reconstituted collagen prepared in various ways and placed under the skin of gunea pigs. In this way a sample could be pretreated, placed in the animal, and then reisolated at a later time for analysis.

113

Major Findings:

Chemical studies on collagen have previously shown that cross-linking is initiated in a specific region of the collagen molecule. A lysyl residue in a non helical region is enzymatically converted to an aldehyde (allysine) which reacts with other functional groups on adjacent polypeptide chains to form covalent cross-links. Since the region is nonhelical and at the amino terminal ends of the α chains of collagen, it can be removed by enzymatic treatment. Chemical studies have also suggested that collagen from lathyritic animals (fed β-aminopropionitrile) differs from normal collagen only in that the conversion of lysine to allysine did not occur. To confirm and extend these results the following experiments were done. (1) Normal collagen was implanted in normal animals. (2) Collagen from lathyritic animals was implanted in normal animals. (3) Normal collagen was implanted in lathritic animals (4) Chymotrypsin-treated collagen was implanted in normal animals. After removal and examination for changes in cross-linking, it was found that normal as well as lathyritic collagen in normal animals continued to cross-link in an essentially normal fashion while normal collagen in lathyritic animals did not cross-link. These results show that cross-linking can proceed in a diffusion chamber where cells, but not enzymes, would be excluded. The defect in lathyrisin is clearly at the enzymatic step in allysine formation. Enzymatic removal of a specific site, previously defined chemically, prevented cross-linking. It is concluded that this site is the major or sole site of cross-link formation during early maturation of collagen.

Significance to Dental Research

The maturation of collagen is fundamental property of collagen necessary for the proper function of connective tissues. These implant studies using carefully controlled conditions will lead to a better understanding of the process and its control. The basic findings will hopefully be useful in determining conditions under which collagen can be used as an implant to promote healing after operative procedures such as tooth extraction.

Proposed Course of Project:

The current studies should be completed within the contract period (Ending December 1968). It is hoped to extend the contract an additional year to study resorption of collagen using the same techniques.

Shoshan, S., and Finkelstein, S.: Cell growth promoting effect of enriched collagen solutions thermally gelled in vivo. Israel J. Med. Sciences 3, 755-58, Sept-Oct. 1967.

Shoshan, S., and Finkelstein, S.: In vivo studies on collagen cross-linking. Biochim. Biophys. Acta. 154, 261-63, Jan. 1968.

Serial No. NIDR-21 (62)
1. Biochemistry
2. Protein Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: The Chemistry of Bone and Cartilage Collagens

Previous Serial Number: NIDR-31

Principal Investigator: Dr. E. J. Miller

Other Investigators: Dr. J. M. Lane, Dr. G. R. Martin, and Dr. K. A. Piez

Cooperating Units: Laboratory of Experimental Pathology, NIAMD, Dr. L. Sokoloff
Laboratory of Histology and Pathology, NIDR, Dr. E. D. Eanes

Man Years:

Total:	4
Professional:	2
Other:	2

Project Description:

Objectives:

To study the biosynthesis of bone and cartilage collagens; specifically, to determine their primary structure, mode of cross-linking, and relationships with other connective tissue components, to study alterations of these parameters in pathological states.

Methods Employed:

Usual laboratory procedures of biochemistry.

Major Findings:

An investigation of the primary structure of collagen in a normally calcifying tissue (chick bone) has been initiated using $\alpha 1$ and $\alpha 2$ chains isolated from acetic acid-soluble collagen of the femora and tibiae of lathyritic chicks. Acid-soluble collagen was used exclusively in these studies since previous results indicated that

Part A (continued)

extraction of bone collagen in denaturing solvents led to degradation of extracted components. Cleavage of the α1 chain (containing nine methionyl residues) at methionyl residues with cyanogen bromide gives rise to ten peptides which have been separated by ion exchange and molecular sieve chromatography. The ten peptides obtained in this manner each constitute a unique portion of the α1 chain as demonstrated by chromatographic properties, amino acid compositions and molecular weight determinations. In addition, the isolated peptides account for all of the amino acids in the α1 chain and their molecular weights totaled 94,000 (as determined by molecular sieve chromatography) in good agreement with 95,000 found by sedimentation studies on α1. These peptides, therefore, represent convenient starting materials for further studies of the primary structure of the α1 chain. Although these results indicate that the α1 fraction of this collagen is a single molecular species, it was found that some heterogeneity is introduced as a result of variable hydroxylation of lysyl residues. Variation in the degree of hydroxylation of lysyl residues was evident from amino acid analyses of most of the peptides containing both lysine and hydroxylysine. For instance, α1-CB3 was comprised of 149 amino acids and contained 4.5 lysyl residues and 0.5 hydroxylysyl residues. This phenomenon was further investigated in one of the smaller peptides, α1-CB1. The latter peptide contained 17 amino acids, was derived from a nonhelical portion of the α1 chain (since it contained only 3 glycyl residues), and appeared to be homologous to α1-CB1 previously isolated from rat skin collagen. Like α1-CB1 of rat skin collagen, α1-CB1 from chick bone collagen contained one lysyl residue, but in the latter case it was consistently observed that the lysyl residue was approximately 50% hydroxylated. Digestion of α1-CB1 with trypsin and isolation of the resulting peptides showed that lysine and hydroxylysine occupied identical positions in the peptide. Preliminary results from the studies on bone collagen suggest that intermolecular cross-linking predominates in bone collagen and that α1-CB1 is somehow involved in their formation, presumably through the lysyl residue. The significance of the partial hydroxylution of the lysyl residue in α1-CB1 of chick bone collagen is, at present, unknown.

In collaboration with Dr. Eanes, NIDR, a study of the x-ray diffraction properties of bone collagen has been completed. These studies were designed to shed light upon the forces responsible for the relative insolubility of bone collagens. The x-ray diffraction data indicates that bone collagen fibrils are much less highly oriented than the fibers of tendon, a result to be expected in view of the random orientation of osteons in secondary bone and the different orientations of fibers within a given osteon. Although helical structure is maintained when the bone collagen is

Part A (continued)

allowed to equilibrate with water or dilute acid solutions, a reversible lateral separation of adjacent molecules does occur as indicated by a shift of the equatorial reflection from a spacing of 11Å (dry) to 16.5Å (wet). When the bone collagen is in contact with denaturing solvents (5 M guanidine hydrochloride or 5 M lithium chloride), a collagen x-ray diffraction pattern is no longer obtained indicating a loss of helical structure and disruption of lateral molecular aggregation. When bone collagen is washed free of denaturing solvents, the characteristic collagen diffraction pattern is restored with no net change in intensity of the lines or degree of fiber orientation. In contrast, rat tail tendon subjected to the same treatment exhibits an irreversible loss of helical structure and intermolecular spatial relationships. The tail tendon will, however, display a stability similar to bone collagen if it is pretreated with formalin solution. These results indicate that bone collagen molecules are stabilized by a high degree of intermolecular cross-linking which provides a number of fixed points regulating the movement of molecules during denaturation and allows a rapid restoration of the spatial relationships between individual chains and molecules during renaturation after removal of the denaturing solvent.

In collaboration with Dr. Sokoloff, NIAMD, studies on human costal and articular cartilage were continued. Specifically, the current studies were designed with a view to characterizing the pigment associated with connective tissues in aging. The yellowish pigment is prominent in cartilaginous tissues such as rib cartilage and has previously been thought to bind to collagen fibers making them more and more insoluble as a function of age. It was found that the pigment resisted extraction in a wide variety of aqueous and organic solvents, but could be brought into solution during proteolytic digestion of aged rib cartilage. Following short periods of proteolytic digestion the pigment remained nondialysable, but was partially dialysable after long term digestion with large quantities of enzyme. These results suggested that the pigment was indeed associated with protein. Moreover, isolation of the acidic polysaccharides from the digestion mixture demonstrated that the pigment was not associated with that fraction of the tissue. Further studies involving trichloroacetic acid fractionation and amino acid analyses of the proteolytic digests demonstrated that the pigment was associated exclusively with non-collagenous protein. It is interesting to note that rib cartilage which displays an unusual amount of pigmentation with age, also accumulates a high proportion of noncollagenous protein as a function of age. On the other hand, articular cartilage which retains a high proportion (approximately 90%) of its protein as collagen throughout life, exhibits very little pigmentation.

Part A (continued)

Significance to Dental Research:

The collagens of bone and cartilage comprise highly significant proportions of the organic material in these tissues. Moreover, it can be assumed that the collagens in these tissues play a role in normal development and function of the tissues. The characterization of bone collagen at the molecular level is expected to shed light upon the possible role of collagen in mineral deposition and similar studies on the collagen in various cartilages will lead to an understanding of the manner in which collagen and protein-polysaccharide interact to provide a highly elastic tissue yet one which is capable of withstanding considerable stress.

Proposed Course of Project:

These studies will be continued with a view toward chemical and structural characterization of bone and cartilage collagen at the molecular level.

Part B Publications:

Miller, E. J., Martin, G. R., Piez, K. A., and Powers, M. J.: Characterization of chick bone collagen and compositional changes associated with maturation. J. Biol. Chem. 242: 5481-5489, Dec. 1967.

van der Korst, J. K., Sokoloff, L., and Miller, E. J.: Senescent pigmentation of cartilage and degenerative joint disease. A.M.A. Arch. Pathol., in press, 1968.

Miller, E. J., and Martin, G. R.: The collagen of bone. Clinical Orthopaedics and Related Research, in press, 1968.

Serial No. NIDR-22(62)
1. Biochemistry
2. Connective Tissue
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: The Chemistry and Biosynthesis of Elastin

Previous Serial Number: NIDR-37

Principal Investigators: Dr. G. R. Martin and Dr. S. R. Pinnell

Other Investigators: Dr. E. J. Miller, Dr. E. Schiffmann and Dr. E. R. Goldstein

Cooperating Units: None

Man Years:

Total	: 4 and 1/4
Professional	: 3
Other	: 1 and 1/4

Project Description:

Objectives:

Collagen and elastin, the major connective tissue proteins, depend on cross-linking for their structural integrity. The purpose of this project is to (1) identify and study the enzyme responsible for initiating the cross-link process and (2) study the biosynthesis of cross-linking by chemically interfering at various levels of the process.

Methods Employed:

Usual laboratory techniques.

Major Findings:

Earlier work in this laboratory established that the initial step in the cross-linking of both collagen and elastin is identical. Lysine in peptide-linkage is converted to alpha-amino adipic-δ-semialdehyde. For the sake of brevity, we have given this compound the trivial name allysine to indicate that it is an aldehyde derived from lysine. The enzyme responsible for this conversion has been detected with the aid of two different assays utilizing an elastin substrate. (1) Elastin labelled with lysine-6-^{3}H releases tritium

120

when lysine is converted to allysine. The tritium released is measured as tritium water following distillation of the water in the reaction mixture. (2) ^{14}C-allysine produced from elastin labelled with lysine-^{14}C can be determined by amino acid analysis of its oxidized derivative ^{14}C-α-aminoadipic acid. With the aid of these two assays, enzyme has been detected in homogenates of skin, aorta and bone. The enzyme is soluble in phosphate-buffered saline, requires no readily dialyzable cofactors, and is inhibited by physiologically active levels of such lathyrogens as β-aminopropionitrile and semicarbazide. The activity is reduced or absent in extracts prepared from the bones of lathyritic chick embryos. The enzyme is distinct from such previously described enzymes as mono or diamine oxidase. The enzyme is specific in that it converts lysine in peptide linkage to allysine but does not act on free lysine.

Previous work has established that β-aminopropionitrile prevents the cross-linking of collagen and elastin at the step where certain lysines in peptide linkage are converted to allysine. Now we have found a compound that inhibits cross-linking after the lysine-allysine conversion.

Penicillamine, a compound previously shown to inhibit desmosine biosynthesis in elastin, has recently been shown to interfere with collagen cross-linking. In an effort to determine the nature of penicillamine interference with cross-link biosynthesis, the effect of penicillamine was studied on the formation of allysine in elastin. Unlike BAPN which interferes with allysine formation, penicillamine interferes with desmosine formation after allysine is formed, causing the accumulation of an elastin with a high aldehyde content.

Significance to Dental Research:

Collagen and elastin, the major structural proteins of the body, constitute an important part of the tissues of the oral cavity. Their structural integrity depends largely upon proper cross-linking. Further knowledge of connective tissue protein cross-link formation may contribute to our understanding of pathological conditions involving connective tissue.

Proposed Course of Project:

Studies on the enzyme responsible for allysine formation from lysine are just beginning. Attempts will be made to purify the enzyme, identify precisely its substrate, determine any co-factor(s), and identify tissue levels in normal and pathological tissues. The effect of penicillamine on collagen cross-link biosynthesis will be studied to determine if the collagen effect

is similar to the effect in elastin.

Part B Publications:

Pinnell, S. R., Martin, G. R., Miller, E. J.: The nature of the inhibition of desmosine biosynthesis by D-penicillamine. Science. In press.

Pinnell, S. R., Martin, G. R.: Cross-linking of collagen and elastin: the enzymatic conversion of lysine in peptide linkage to α-aminoadipic-δ-semialdehyde by an extract from bone. Proc. Natl. Acad. Sci. U. S. In press.

Miller, E. J., Martin, G. R., Piez, K. A., and Powers, M. J.: Characterization of chick bone collagen and compositional changes associated with maturation. J. Biol. Chem. 242:5481-5489, Dec. 1967.

Miller, E. J. and Martin, G. R.: The collagen of bone. Clinical Orthopaedics and Related Research, 1968. In press.

Serial No. NIDR-23(62)
1. Biochemistry
2. Connective Tissue
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Calcification of Organic Matrices

Previous Serial Number: NIDR-34

Principal Investigators: Dr. E. Schiffmann, Dr. D. R. Lavender

Other Investigators: Dr. E. J. Miller and Dr. G. R. Martin

Cooperating Units: None

Man Years:

Total:	4
Professional:	2
Other:	2

Project Description:

Objectives:

To study basic mechanisms whereby organic matrices calcify. The systems under investigation are: 1. mineralizing elastin-containing tissue in vitro. 2. calcification in embryonic chick bone rudiments. 3. cultures of cells from osteoid and related tissue.

Methods Employed:

1) Elastic Tissue

Nuchal ligament preparations, a material rich in elastin, were obtained from exhaustive extraction of the bovine tissue with guanidine to remove much collagenous component. Mineralization of this matrix was accomplished in calcifying media in the presence of Ca^{45} and/or P^{32} at 38°. Extent of calcification was determined by isotopic assay on aliquots of acidic extracts of the mineralized matrix. Measurement of mineral formation in the absence of matrix was by assaying the radioactivity of aliquots of an acidic solution of mineral that had been collected on a Millipore filter. Labelling matrix with Fe^{55} was accomplished by incubating sulfhydryl-treated elastin in the presence of 0.01 mM $Fe^{55}Cl_3$. Incubation of matrix with elastase was carried out in Tris at 38°, pH 7.5. Standard procedures were used to determine amino acid contents of samples.

2) Chick Bone Rudiment Calcification.

Chick tibias were isolated from embryos of varying ages and placed in organ culture dishes together with tissue culture media under a 95% air-5% CO_2 gas phase. Ca^{45} and proline-H^3 were added as needed to medium. Calcium uptake by rudiments was determined by both a fluorometric method for calcium and by assaying radioactivity of acid extracts of the rudiments. Proline incorporation was measured by isotopic assay.

3) Bone Cell Culture

Cells were obtained from embryonic chick bones, rat and human bones after trypsinization. These were grown in tissue culture medium under 95% air-5% CO_2.

Major Findings:

1) Elastin-containing tissue

It has been shown that Fe^{3+}, complexed to sulfhydryl groups of the matrix, participates in the nucleation of an elastin matrix. Studies on the characterization of amino acids at or near the nucleation site have been carried out, and it has been determined to which residues Fe^{3+} is bound. In addition some information has been obtained on the nature of the nucleating complex in a calcifying medium in the absence of a matrix.

a) Amino acids at the nucleating site.

Proteolytic enzymes have been used as probes to determine those amino acids concentrated at the nucleation site on mineralizing elastin. We have assumed that the deposition of mineral at specific sites in elastin would prevent the enzymatic degradation of these regions by proteases. Elastase was found to remove up to 97% of the organic phase of mineralizing elastin without appreciable solubilization of calcium. The composition of the organic material associated with the mineral phase was quite distinct from the original elastin as well as residues remaining after the digestion of non-mineralized samples. It was found that calcified residues were greatly enriched in cysteine as well as dicarboxylic amino acids. These studies indicate that only a small portion of this matrix is associated with mineral. The portion of the matrix associated with the mineral phase is distinct from the rest of the matrix and is rich in cysteine which was shown previously to be involved with nucleation.

b) Fe^{3+} binding specificity.

The amount of Fe^{3+} bound as measured by the use of Fe^{55} was greater in calcified residues compared to that in uncalcified residues, and this increased binding could be accounted for by ascribing it to sulfhydryl groups. It is estimated that 1 mole of Fe is bound per mole of sulfhydryl, and less than 0.05 mole of Fe is bound per mole of other amino acids.

Therefore, evidence is presented for the presence of both cysteine and Fe^{3+} at the nucleating site in mineralizing elastin.

c) The nucleating complex in solution.

It was conceivable that a complexed metallic cation nucleated by binding phosphate ions to its unfilled coordination positions. In a calcifying medium, calcium ions might then be attracted to the complex, initiating nucleation. The formation of a ternary complex between a cation (Fe^{2+}), a complexing agent (norepinephrine), and phosphate was studied by two methods. Potentiometric titration of solutions of cation and complexing agent and solutions containing cation, ligand, and phosphate indicated that in both cases the cation-ligand complex was present. Also a typical color formation indicated the formation of this complex. In the solution without phosphate, the amount of complex that precipitated was greater than in the solution that contained phosphate. This was demonstrated by absorption spectrophotometry of the supernatents of the two solutions. The results suggested that phosphate solubilized to some extent the cation-ligand complex perhaps by forming a ternary complex. It was also shown that accelerated formation of a mineral phase occurred at a much greater rate in calcifying solutions containing both cation and ligand than in media with either cation or ligand alone. The evidence suggests that a ternary complex is involved in nucleation of a mineral phase from solution.

2) Mineralization of embryonic chick bone rudiments.

The normal pattern of growth and mineralization of the long bones of the chick embryo has been determined as a basis of comparison for the bones in vitro. Little calcium is found in the chick bone prior to the eighth day, after which the total calcium increased markedly. Yet there is no abrupt change in concentration of calcium in the bone as rapid bone growth accompanies mineralization. However, some important morphological alterations occur in the chick bone as the bone differentiates from a cartilaginous to a true bone structure. This latter change seems to be correlated with cell type as well as mineralization.

In vitro the 9 or 10 day old chick tibia undergoes rapid growth. A

greater growth rate and a higher level of calcium are found in the 9 day old bone grown in medium supplemented with chick embryo extract. In the 10 day old bone, growth is again stimulated by the medium containing embryo extract. However, an equivalent uptake of calcium occurs in bones at lower growth rates in media lacking embryo extract. The levels of calcium accumulating in the 9 day bone grown in the embryo extract-fortified medium or in the 10 day bones grown with or without embryo extract are similar to the levels found in developing bones *in vivo*.

3) *Bone cell culture*.

Two distinct types of cells have been obtained in culture from 8 or 9 day old bone, fibroblasts, and cartilage cells. An additional cell type occurs in cultures obtained from 10 day old bones. These cells arise from the shaft rather than from the ends of the bones. This cell type also can be isolated and cultured from younger bones grown in culture for a few days.

These findings indicate that mineralization occurs as a result of differentiation. A cell distinct in morphology from fibroblast or cartilage cell arises at the site and at the time of mineralization.

Significance to Dental Research:

The processes whereby osseous and non-osseous tissue mineralize are not yet understood. Studies on systems which calcify *in vitro*, such as elastin-containing tissue on one hand and chick bone rudiments as well as isolated cells from the latter source on the other hand may contribute to understanding pathological and normal calcification respectively.

Proposed Course of Project:

1) *Elastin*. The nucleating properties of peptide material from the nucleating site will be studied. It is of interest to know the role, if any, of other functional groups besides sulfhydryl in nucleation and/or crystal growth. With reference to this it may be possible to relate composition of acids at the nucleating site with changes in the composition of the mineral phase.

2. *Chick bone rudiments*. The *in vitro* mineralizing chick bone system will be used to define the factors involved in mineralization.

3. *Bone cell culture*. The ability to culture cells from bone will permit the measurement of cellular activities such as collagen synthesis and cross-linking in cells from normal and diseased tissue.

126

Honors and Awards:

Dr. Dick R. Lavender. Edward H. Hatton Award, IADR, 1968, 1st Place, Graduate Division.

Publications:

Schiffmann, E.: Remarks in First Conference on Biology of Hard Tissues, June 1965. New York Academy Science 1967.

Schiffmann, E., Martin, G. R., and Miller, E. J.: Matrices that calcify in Biological Calcification, Ed. R. Schraer, Appleton-Century-Crofts. In press.

Serial No. NIDR-24(61)
1. Biochemistry
2. Connective Tissue
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Long Term Effects of Water Fluoridation in Grand Rapids, Michigan

Previous Serial Number: NIDR-40

Principal Investigator: Dr. E. Schiffmann

Other Investigators: Dr. F. J. McClure and Dr. G. R. Martin

Cooperating Units: Albert Einstein College of Medicine, Yeshiva University - Dr. E. F. Geever
Grand Rapids-Kent County Health Dept, Grand Rapids, Michigan - Mr. Harold E. Samuelson

Man Years:

Total:	1
Professional:	0
Other:	1

Project Description:

Objectives:

To determine long term effects of fluoridation by histological and chemical examination of rib, vertebra and aorta of individuals residing for at least 20 years in Grand Rapids, Michigan.

Methods Employed:

Grand Rapids, Michigan has been fluoridated since January 1945. To evaluate the long-term effects of fluoride, rib, vertebra and aorta are being collected from the following age categories of postmortem: 0-20, 21-40, 41-60, 61-80 and above 80 years of age. Specimens are sent in fixative to Dr. E. F. Geever of the Albert Einstein College of Medicine, New York, N. Y., for histological examination (Contract PH43-66-941 with Albert Einstein College of Medicine of Yeshiva University, New York, N. Y.; approximate cost $9,500). Portions of the same specimens will be analyzed for fluoride, calcium, phosphorus and ash content. Control specimens are being obtained from New York City and Albany, New York.

128

Major Findings:

Sample collection and histological examination is well advanced and chemical analysis has begun. However, data are not yet sufficient for conclusions to be made.

Proposed Course of Project:

The current contract runs to July, 1968, but will be extended for six months to one year to complete study.

Significance to Dental Research:

Although the long-term effects of fluoride naturally present in water have been studied, the availability of a population receiving controlled amounts of fluoride for more than 20 years offers an unusual opportunity to demonstrate the safety of water containing fluoride at an optimal level for caries inhibition.

Part B: Not included.

Serial No. NIDR-25 (52)

1. Biochemistry
2. Enzyme Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Chemistry and Mechanism of Action of (1) Chymotrypsin C and (2) Transglutaminase

Previous Serial Number: NIDR-36

Principal Investigator: Dr. J. E. Folk

Other Investigators: Dr. H. Kato and Dr. R. L. Boothe

Cooperating Units: Biometrics Research Branch, NHI, Dr. J. P. Mullooly

Man Years:

Total:	4 1/2
Professional:	3
Other:	1 1/2

Project Description:

Objectives:

The purpose of these studies is to examine the basic mechanisms of enzyme action. These include the order of substrate addition and product release, the role of metals in activation, the specific functional groups involved in substrate binding and in catalytic modification, the nature of the amino acid sequence around functional groups, and the effect of protein modification on activity.

Methods Employed:

1. Chymotrypsin C has been labeled at the "active site" histidine residue by means of the specific irreversible inhibitor, ^{14}C-tosyl-L-leucyl chloromethylketone, which was synthesized in this laboratory. The labeled enzyme has been subjected to digestion by pepsin. The resulting labeled peptide has been separated and the amino acid sequence analysis has been carried out by conventional methods.

2. The enzymatic activity of purified transglutaminase has been

studied by kinetic procedures with particular emphasis on the role of metal ion in the hydrolysis reaction. The enzyme has been subjected to inactivation by trace quantities of copper ion. The mechanism of inactivation has been studied using sulfhydryl titration technics and ^{14}C-labeling methods. A number of peptide derivatives containing methyl glutamine residues have been prepared in an effort to examine the steric arrangement of groups in the substrate binding site of transglutaminase. The enzyme has been subjected to N-terminal and C-terminal analysis with the hope of determining the number of polypeptide chains in the molecule.

Major Findings:

1. The complete sequence of 12 amino acids around the active histidine of chymotrypsin C has been elucidated. This sequence, Ala-Ala-His-Cys-Ile-Asp-Ser-Gly-Thr-Ser-Arg-Thr, shows certain differences from those around active histidines in chymotrypsins A and B.

2. Kinetic and chemical studies strongly support a premise that the divalent cations, calcium and strontium, activate transglutaminase by binding at two separate sites in the enzyme molecule and that a conformational change in the enzyme protein accompanies the metal ion activation. These studies also form the basis for a mechanism of metal ion and substrate additions, termed a "ping pong" mechanism, wherein a common acyl enzyme intermediate is formed during the hydrolysis or transfer reactions and where water or acceptor amine, respectively, add after the release of ammonia.

   The findings that copper ion-catalyzed inactivation of transglutaminase requires high levels of calcium ion, results in essentially no binding of copper, but causes the formation of two disulfide bonds suggests that inactivation occurs only in the conformationally changed enzyme molecule and is a result of limited oxidization of 4-sulfhydryl groups. Preliminary studies also suggest that the so-called "active site" sulfhydryl group is not involved in disulfide bond formation during this inactivation. The fact that this inactivation is readily reversed by reducing agents is consistent with the above findings.

   Preliminary studies show that the length of the methylene chain in transglutaminase substrates is not limited to that of glutamine - the α-amino-adipamyl derivative is a substrate for the enzyme. First findings with N-terminal and C-terminal amino acid methods show that this enzyme molecule of 90,000 molecular weight has available terminal groups. The type and number of residues have not been assigned as yet.

131

Significance to Dental Research:

Understanding of the mechanisms of action of enzymes, the use of these enzymes for protein modifications and structure studies, and a knowledge of the functions and interrelationships of these enzymes contribute to the resolution and understanding of basic biochemical processes.

Proposed Course of Project:

1. Further studies of the primary structure of chymotrypsin C and comparisons of this structure with those in other proteolytic enzymes are underway. The proposed course of investigation is a study of zymogen activation with particular emphasis on structural changes.

2. Future studies of transglutaminase will entail an examination of the location and spacial arrangement of the sulfhydryl groups that are involved in disulfide formation during copper ion-catalyzed inactivation. The object of this investigation is to obtain more information concerning the calcium ion-induced conformational rearrangements in the molecule.

   Stereochemical substrates and irreversible inhibitors are in preparation. A study of the action of transglutaminase on these substrates and inhibition by these inhibitors should provide information as to the type of amino acid side chains in or near the binding site of the enzyme and the orientation of the substrate on the enzyme surface.

   Studies are in progress on the number and type of terminal residues in transglutaminase. Various reagents are under test as possible chemical modifying agents.

Part B Publications:

Folk, J. E., Cole, P. W., and Mullooly, J. P.: Mechanism of action of guinea pig liver transglutaminase IV. The trimethylacyl enzyme. J. Biol. Chem. 242: 4329-4333, October 1967.

Folk, J. E., Cole, P. W., and Mullooly, J. P.: Mechanism of action of guinea pig liver transglutaminase V. The hydrolysis reaction. J. Biol. Chem. 243: 418-427, Jan. 1968.

Tobita, T., and Folk, J. E.: Chymotrypsin C III. Sequence of amino acids around an essential histidine residue. Biochim. et Biophys. Acta 147: 15-25, July 1967.

Folk, J. E.: Carboxypeptidase A (bovine pancreas). In Specifications and Criteria for Biochemical Compounds, (Ed. 2), Natl. Acad. of Sciences, 1967, pp. 233-234.

Folk, J. E.: Carboxypeptidase B (porcine pancreas). In Specifications and Criteria for Biochemical Compounds, (Ed. 2), Natl. Acad. of Sciences, 1967, pp. 235-236.

Folk, J. E.: Chymotrypsin A (bovine pancrease). In Specifications and Criteria for Biochemical Compounds, (Ed. 2), Natl. Acad. of Sciences, 1967, pp. 237-238.

Folk, J. E.: Trypsin (bovine pancreas). In Specifications and Criteria for Biochemical Compounds, (Ed. 2), Natl. Acad. of Sciences, 1967, pp. 261-262.

Folk, J. E.: Transglutaminase (guinea pig liver). In Tabor, H., and Tabor, C. W. (Eds.): Metabolism of Amino Acids and Amines. In press.

Serial No. NIDR-26(63)
1. Biochemistry
2. Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Prenatal Developmental Factors Influencing Oral Disease

Previous Serial Number: NIDR-37

Principal Investigator: Dr. A. J. Steffek (ADA Research Associate)

Other Investigators: Dr. M. V. Barrow and Dr. A. C. Verrusio

Cooperating Units: Office of International Research, NIH, Rio de Janeiro, Brazil, Dr. C. T. G. King

Man Years:

Total:	7 and 1/4
Professional:	3
Other:	4 and 1/4

Project Description:

The Pharmacology Section, LB, NIDR, has been involved during recent years with investigations related to the mechanisms involved in experimentally-produced congenital malformations with particular emphasis directed toward the pathogenesis of cleft palate. Specific teratogens such as chlorcyclizine and related structural analogues, Vitamin A, 6-aminonicotinamide and more recently lathyrogenic agents have been implemented in various laboratory animals in an attempt to define the etiological factors related to the production of these malformations. The results of these investigations are presented as follows:

Subproject A:

Mechanisms involved in normal development of the secondary palate.

Objectives:

Current theories of cleft palate pathogenesis hold that either the palatal shelves fail to assume the horizontal position, or, that having done so, they fail to fuse. Thus it may be of great importance to understand the biological processes underlying both the "rotation" of the shelves to the horizontal position and the subsequent fusion of the shelves. Since the two processes do not

occur simultaneously, they can theoretically be considered separate phases of palate development--each subject to experimental study and manipulation. The tongue may also play a role in palate closure once it has been displaced from between the shelves, by aiding fusion (flattening the shelves and bringing their free edges together).

Methods Employed:

Embryos undergoing palatogenesis were obtained by laparotomy from pregnant rodents (gestational days 14-17). The embryonic heads were then subsequently prepared for conventional histological and histochemical techniques.

Major Findings:

Palatal Closure:

Prior to the initiation of palate closure in the rodent the cranial base is flexed in the area of the craniopharyngeal canal (day 13 1/2 in the mouse and day 15 1/2 in the rat). As the process of palate closure progresses, there is a gradual reduction in the flexure of the cranial base until the palatine processes are horizontal, at which time the cranial base is straight. Rapid growth and straightening of the cranial base might play a role in palate closure by providing the "internal shelf force." Several pieces of evidence support this hypothesis: rat embryos possess a considerable flexure, and their palatal development is not affected by cortisone; the angle of flexure is smaller in C57BL mice than in rats and 17-18% of C57BL embryos have cleft palate after cortisone treatment; and finally, embryos of the A/J strain have almost no range of movement because the cranial base is almost fully extended before palatal closure commences and as a result they have a cleft palate frequency of 100% after cortisone treatment.

Several C57BL/6 embryos have been observed with spontaneously occurring severe micrognathia and microglossia. In those animals with large remnants of the tongue, the palate is cleft and the small tongue extends upwards between the palatine shelves. However, in a few cases the mandible is reduced to the point of being non-existent and there is no tongue. In these animals the palate is fused--although it could hardly be called a "normal" palate. This indicates that the palate can form, even in the absence of the tongue.

Palatal Fusion:

The hydrolytic enzyme acid phosphatase was assayed histochemically

J 35

in the midpalatal oral-facial region of normal A/J mice and Sprague-Dawley rats during fusion of the palatal processes. Enzyme activity was associated with the degenerative epithelium of the midpalatal region in normal 15 and 16 day A/J mouse embryos. The palatal midline epithelium and underlying mesenchyme in normal 16 day rat embryos showed minimal amounts of acid phosphatase. Greatest enzyme activity occurred on the 17th gestational day, associated with degenerative changes in the midline epithelium. By the 18th day, the midline seam was virtually absent and the enzyme activity was principally associated with palatal osteogenesis. These results show that acid phosphatase is present in the midpalatal epithelium during normal palatal fusion of rodent embryos and participates in the breakdown of these opposing epithelium.

Significance to Dental Research:

These investigations have attempted to elaborate mechanisms involved in normal palatal development and have yielded information relevant to other structures of the craniofacial complex, which might potentially participate in the production of experimental cleft palate.

Proposed Course of Project:

Sagittal sections of rat embryos treated with 80 mg/kg chlorcyclizine on days 10-15 of gestation will be examined and the angle of flexure of the cranial base will be compared with that of normal, untreated animals before, during, and after palatal closure to ascertain if the cranial base is affected by this teratogen. Mice of the A/J and C57BL strains will be treated with 19 mg/kg of 6-AN on day 13 1/2 of gestation and then compared with normal embryos to see if cranial base morphology is affected in both these strains--the resistant C57BL and the susceptible A/J.

Subproject B:

Teratogenic agents and congenital oral-facial malformations in mammalian embryos.

Objectives:

To investigate possible mechanisms involved in the production of oral-facial lesions by specific teratogenic agents in various mammalian species.

Methods Employed:

Fetuses obtained from pregnant mice, rats and rabbits treated with lathyrogens or 6-aminonicotinamide during organogenesis were

136

observed for gross congenital malformations. The heads were subsequently removed, fixed, embedded and sectioned by conventional techniques for the histological characterization of the oral-facial lesions.

Major Findings:

A) Lathyrogenic agents

The maternal ingestion of a 50 percent ground Lathyrus odoratus pea seed diet by pregnant Sprague-Dawley rats from the 10th through 20th days of gestation resulted in resorptions and multiple fetal malformations of the limbs, vertebral column and spinal cord and maxillofacial regions including micrognathia and cleft palate. Various degrees of edema were also exhibited in these fetuses. Pregnant mice of the A strain maintained on the same diet from the 9th to the 18th days of gestation produced young with cleft palate and malformations of the vertebral column and spinal cord. The lathyrus factor, β-aminopropionitrile (BAPN) was also checked for teratogenic activity in the rat. Oral administration of this agent (200 mg from the 12th to 19th gestational days) produced a high incidence of resorptions (95%). The viable young that were obtained by this procedure showed edema, cleft palate and vertebral and limb malformations. As the oral dosage of this compound was increased to 500 mg and restricted to the 15th day of gestation, the number of resorptions was markedly reduced to 9%, while cleft palate was observed in 95% of the fetuses accompanied by a variable incidence of edema and limb malformations.

The susceptibility of the rabbit embryo to the teratogenic action of BAPN was also investigated and gross malformations of the limbs and oral-facial region including palatal clefts were again demonstrated. This latter finding indicates that the teratogenic susceptibility of the compound is not limited to the rodent species.

The oral administration of other lathyrogenic agents (D-penicillamine, aminoacetonitrile and semicarbazide) to pregnant rats during the period of organogenesis also resulted in the production of congenital malformations involving the oral-facial regions. Cleft palate was observed in a high incidence of the viable young obtained from pregnant rats treated with 400 mg of D-penicillamine from the 10th through 15th days of gestation. The oral administration of aminoacetonitrile in half the amount (200 mg) of either BAPN or D-penicillamine at comparable times of gestation resulted in maternal deaths indicating a greater toxicity for this compound. As the dosage was reduced to 50 mg and administered only on the 15th day of gestation (period of palatal closure) the viable young obtained from this procedure all demonstrated clefts of the secondary palate. Virtually no resorption sites were noted during this

regime. Semicarbazide administration at the same dose as aminoacetonitrile (50 mg) to pregnant rats from the 10th through 16th gestational days also resulted in the production of cleft palate in the offspring obtained from this treatment.

The histopathological characterization of the oral-facial lesion of rat embryos observed after these treatments were as follows:

1) The palatal processes retained their vertical orientation and exhibited a moderate degree of hypoplasia, although in some instances, horizontal transmigration of the palatal shelves had been effected. In the instance in which the vertical orientation of the palatal processes persisted, no histological evidence of glossopalatal fusion was observed. Another histological feature of the oral-facial lesion obtained with these treatments was a mass of well defined heterotopic cartilage associated with Meckel's cartilage and directed medially near the base of the tongue. This finding shows that lathyrogenic agents have the capacity of producing heterotopic cartilage in the developing oral-facial regions of rat embryos.

B) 6-Aminonicotinamide

A colony of mice was established to investigate a cytoplasmically transmitted difference in response to the teratogenic effects of 6-aminonicotinamide (6-AN) in the C57BL and A/J strains. The frequency of cleft palate produced by maternal treatment on day 13 1/2 of gestation with 19 mg/kg 6-AN is lower in C57BL (20%) than in A/J (100%) mice. The frequency was found to be significantly lower in the offspring of C57BL females maintained on Purina Lab Chow (20%) than on Breeder Chow (70%). A/J females do not show the effect of diet. There is a matroclinous reciprocal cross difference in the frequency of induced cleft palate which persists in the back cross, but only when the F_1 mothers are maintained on Lab Chow. Since mitochondria are transmitted through the egg cytoplasm, and 6-AN forms an inactive NAD analogue that interferes with oxidative phosphorylation in mitochondria, it is reasonable to postulate that the cytoplasmic factor is associated with a difference in the mitochondria of the two strains.

The important difference between the two diets used seems to be the higher niacin content in Lab Chow, since niacin counteracts the effect of 6-AN. Because there were other differences between the diets and commercial diets are highly variable in content, the experiments are currently being repeated using a defined diet supplemented with two different levels of niacin (45.4 mg/lb and 20 mg/lb). Preliminary results show that the cytoplasmic effect is still present on these diets and can be directly related to the

level of niacin in the diet.

Significance to Dental Research:

These results extend out studies in the pathogenesis of experimentally-induced cleft palate. The ability to produce predictively specific types of palatal defects in various mammalian species offers an excellent technique for investigating drug-induced oral-facial malformations in particular and gene-environment interactions in general.

Proposed Course of Research:

A) Lathyrogens

Quantitative biochemical investigations will be undertaken in an attempt to relate the palatal and cartilaginous lesions with defects of connective tissue metabolism.

B) 6-Aminonicotinamide

The mitochondrial difference described above will be further investigated by an electron microscopic study of the mitochondria of the two strains before and after treatment with 6-AN. It is hoped that the mitochondria of the two strains will present a different morphological reaction to the administered teratogen, and that this response will help elucidate the nature of the mitochondrial difference.

Subproject C:

Intrauterine application of teratogens.

Objectives:

To develop a technique for intrauterine application of compounds for assessing the role of maternal and placental metabolism, the teratogenic compound, and the critical time in the production of fetal rat malformations by benzhydrylpiperazine compounds. Additionally, structural analogues of chlorcyclizine (relatively unaltered by maternal metabolism) were presented to the developing embryo to investigate the minimum structure eliciting cleft palate and syndactyly.

Methods Employed:

Pregnant Sprague-Dawley rats (11th to 16th days of gestation) were anesthetized, a mid-line abdominal incision made and the uterus was exposed. A small cut was made in the uterine wall and a

Millipore filter (0.2 cm x 0.2 cm) impregnated with 50 μg chlorcyclizine HCl, 50 μg norchlorcyclizine HCl, or a HCl control was placed on either the intact amniotic sac (over the fetus) or the placenta. The uterus was returned to the abdominal cavity, the incision closed, and gestation continued to the 20th day. At this time the maternal animal was killed and the fetuses were examined. After insertion each compound was traced by radioactive (chlorcyclizine) or spectrophotometric (norchlorcyclizine) assay of fetal, placental or maternal tissues. The other structural analogues chemically related to chlorcyclizine tested for their teratogenic potential by this technique were by generic name: homochlorcyclizine, norhomochlorcyclizine, chlorcyclizine-N-oxide, cyclizine, norcyclizine, and others by formula name, piperazine, 4 chlorbenzhydrol, (4 chlorphenyl-1-methyl) N-piperazine, (2 methy phenyl phenyl methyl) piperazine, (2 methyl phenyl phenyl methyl) N methyl N piperazine, and (4 chloro diphenyl methyl) tris methyl methyl piperazine.

Major Findings:

Filters containing the HCl control, those inserted on the placenta and those implanted other than day 13 or 14 produced no cleft palate. A specific left fore limb syndactly was produced when filters impregnated with norchlorcyclizine, and to a lesser degree chlorcyclizine, were inserted centrally over the fetus on days 11 through 16. Left hind limb syndactly was seen when norchlorcyclizine filters were placed off center over the hind limb on the 14th day.

When the filters were inserted on the amnion, levels of 20-40 μg/g chlorcyclizine or norchlorcyclizine were found in the fetus, but when they were placed on the placenta only 5-10 μg/g were found in the fetus. These concentrations were maintained for two hours, but after eight hours neither compound could be detected in the uterine area. Inserted chlorcyclizine was not demethylated to norchlorcyclizine by the fetus or by the placenta. These data suggest that norchlorcyclizine is the more potent teratogen, the critical time for cleft palate production is the 13th or 14th day (11th through 16th days of gestation for syndactly production), and that these compounds act directly on the fetus in producing these malformations.

The analogs that produced cleft palate were: norhomochlorcyclizine (at a rate of 33%), (2 methyl phenyl phenyl methyl) N piperazine (13%), homochlorcyclizine (10%), norcyclizine (7%) and (4 chloro diphenyl methyl) tris methyl methyl N piperazine (2%). These compounds also caused specific limb syndactly in parallel percentages. In addition, the compounds cyclizine and (2 methyl phenyl phenyl methyl) N methyl N piperazine produced 2% and 4% syndactly respectively.

Significance to Dental Research:

Direct access to the developing fetus with small quantities of teratogens and the elimination of the factors of maternal metabolism and excretion of these compounds can greatly aid the investigation of the pathogenesis of cleft palate. Minimal structural activity in eliciting the teratogenic response can also be assessed by this technique.

Proposed Course of Research:

An attempt to alter the teratogenic response of norchlorcyclizine will be made by co-inserting possible competitively binding non-teratogenic compounds such as structural analogs or Ca salts.

Previous in vitro studies have shown that norchlorcyclizine binds stoichiometrically to bovine nasal septum cartilage. When calcium concentration is high, norchlorcyclizine is displaced completely whereas if the calcium concentration is low norchlorcyclizine preferentially binds to the cartilage. These in vitro cartilage binding studies will be expanded to include all the mentioned analogs and an analysis of their degree of binding with norchlorcyclizine and calcium displacement will be undertaken. This data will indicate if a correlation exists between this binding affinity of cartilage and expression of teratogenicity.

Subproject D:

Different effects of chlorcyclizine in the Sprague-Dawley and Long-Evans strains of rats.

Objectives:

To compare the teratogenic effect of chlorcyclizine in the Long-Evans and Sprague-Dawley strain of rat.

Methods Employed:

Pregnant Long-Evans and Sprague-Dawley rats were treated with either 40 mg/kg, 60 mg/kg or 80 mg/kg of chlorcyclizine on days 12 through 15 of gestation. On day 20 mothers were killed and fetuses examined grossly for edema and cleft palate. Each litter was divided and half the fetuses placed in Bouin's fluid for later internal examination by razor blade hand sections and half in alcohol for clearing and alizarin staining.

Major Findings:

At a dose of 40 mg/kg the main differences in the response of the

two strains was a 20% incidence of cleft palate in the Sprague-Dawley (SD) versus a 5% incidence in the Long-Evans (LE) with half of the SD clefts being fused to the tongue. Differences became more apparent at a dose of 60 mg/kg where hydrocephalus and hydronephrosis were more common in the SD strain. In addition decreased or absent sternal and central vertebral body calcification and mild edema occurred only in the Sprague-Dawley strain. At 80 mg/kg severe edema, all fused cleft palates, absent sternal, central vertebral body and sacral calcification occurred only in the Sprague-Dawley strain whereas minimal edema, unfused cleft palate and mostly decreased rather than absent central vertebral body calcification was noted in the Long-Evans group.

These results indicate a definite difference in response to chlorcyclizine with the Sprague-Dawley strain being more sensitive to the drug.

Proposed Course of Project:

Comparative metabolism studies in both strains will be carried out to elucidate whether the difference in response is due to a more rapid turnover of chlorcyclizine, less production of norchlorcyclizine, the active metabolite, or some other mechanism.

Significance to Dental Research:

This study will further delineate the differences that are manifest in the oral regions of two strains of rats subjected to the same teratogen.

Subproject E:

Prolonged gestation in the rat as an aid in teratological research.

Objectives:

To evaluate whether prolonging gestation in rats may be helpful in elucidating certain teratogenic mechanisms.

Methods Employed:

Groups of pregnant rats received orally one of the following regimens: chlorcyclizine, 80 mg/kg, gestational days 10-15; Vitamin A, 100,000 units 10-15; β-aminopropionitrile 200 mg days 10-16 or Lathyrus odoratus ground seed in the diet days 10-20. Litters were killed on day 20 or on day 23 after prolonging gestation with progesterone (10 mgIM, days 20-22). Untreated animals served as controls.

Major Findings:

Observations concerning teratogenic effects of several compounds have been made in rat litters after prolonged gestation. In the 23 day chlorcyclizine group, limb abnormalities, edema and hydronephrosis were more marked than in the 20 day groups. Vertebral calcification usually not present on day 20 had occurred by day 23 whereas cryptorchidism persisted. After Vitamin A limb and ear abnormalities were more easily identified and prognathism was more severe on day 23. BAPN and Lathyrus induced limb malformations noted on day 20 tended to progress in severity by day 23. Prolonging gestation avoids early death of deformed fetuses, permits fuller development of certain induced abnormalities and allows ambiguous findings to be further clarified. It also may help determine whether an unusual observation is the result of delayed development and therefore transient or is a more permanent defect.

Significance to Dental Research:

This study can investigate "postnatal" changes in the oral-facial region due to specific teratogens by the prolongation of gestation. Specifically, the extensive chondrogenesis present throughout the oral-facial regions can be observed by prolongation of gestation for potential ossification by maintaining the fetus *in utero*.

Proposed Course of Research:

Detailed measurements of the mandibular growth after Vitamin A administration in excess and after prolonged gestation will be made; in addition further clarification of delay or absence of calcification of the central vertebral bodies after chlorcyclizine will be attempted by evaluating the degree of calcification on days 20, 21, 22 and 23.

Part B Publications:

Steffek, A. J., King, C. T. G. and Wilk, A. L.: Abortive effects and comparative metabolism of chlorcyclizine in various mammalian species. *J. of Teratology*. In press.

Koziol, C. A. and Steffek, A. A.: Acid phosphatase activity in palates of developing normal and chlorcyclizine treated rodents. *Archives of Oral Biology*. In press.

Fraser, F. C., Chew, D. and Verrusio, A. C.: Oligohydramnios and cortisone-induced palate in the mouse. *Nature* 214:417-418, 1967.

Verrusio, A. C., Pollard, D. R., and Fraser, F. C.: A cytoplasmically transmitted diet-dependent, difference in response to the teratogenic effects of 6-aminonicotinamide in the mouse. Science 160:206-207, 1968.

Serial No. NIDR-27(66)
1. Biochemistry
2. Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Study of Teratogenesis and Organogenesis in the Non-human Primate (Contract 43-66-457, Hazelton Laboratories, Falls Church, Virginia)

Previous Serial Number: NIDR-39

Principal Investigator: Dr. A. J. Steffek (ADA Research Associate)

Other Investigators: Dr. K. A. Piez, Dr. M. V. Barrow

Cooperating Units: Office of International Research, NIH, Rio de Janeiro, Brazil, Dr. C. T. G. King

Man Years:

None

Project Description:

Subproject A:

Thalidomide syndrome in Rhesus monkeys (Macaca mulatta).

Objectives:

To study the response of the Rhesus monkey to thalidomide and further delimit the teratogenic time period.

Methods Employed:

This project is conducted by Contract PH43-66-457, 1967, to Hazelton Laboratories, Falls Church, Virginia. The principal investigators at Hazelton Laboratories are Dr. William M. Reese, Jr., and Mr. Howard Feinman. The Project Officer is Dr. K. A. Piez and the cost of the contract is 37,000. The Rhesus monkeys were maintained at the Hazelton Facility and the fetuses are obtained by Caesarean Section. The experimental procedures are performed at Hazelton Laboratories and subsequent examination and analysis of the specimens is executed at NIDR.

Major Findings:

Three pregnant Rhesus monkeys (Macaca mulatta) were treated with 100 mg of thalidomide by intubation on the following schedules: mother 286L - days 24, 25, 26 of gestation; mother 197N - gestational days 26, 27, 28; and mother 140R - gestational day 30 only. The fetus from mother 286L showed severe phocomelia of all extremities, dislocated shoulder and hip joints, rudimentary digits, micrognathia, a double gall bladder, malrotation of the gut and a double right coronary artery. Mother 197N's fetus showed only a missing first toe of the right foot and an extra digit of the left. The fetus from mother 140R was normal.

Not only is administration during a critical and early time period necessary for the development of severe and complete phocomelia in the Rhesus monkey but an impressively low dosage of the compound is needed to achieve this end. During days 24 to 26 of gestation, no or very early limb buds are present and treatment during this stage of development results in the full phocomelia syndrome. Treatment later (days 26-28) resulted in only posterior limb anomalies indicating a cephalocaudal gradient of susceptibility. By day 30, still before the appearance of finger rays thalidomide was no longer effective under the conditions of our experiment.

Thus the primary site of action on the extremities must lie in its effect on early mesenchymal tissue destined to become or influence the proximal portions of the limbs.

Soft tissue malformations both typical (gut malrotation) and atypical (a double gallbladder rather than an absent one) for thalidomide were also demonstrated.

Significance to Dental Research:

The oral-facial embryogenesis of the non-human primate simulate more closely the human as compared to rodents. This fact could allow a more valid extrapolation of teratogenic effects based on human experience, including those malformations of the oral-facial regions.

Proposed Course of Project:

Additional investigations evaluating the non-human primate as a possible model system in the assessment of other potential human teratogens will be initiated with particular direction towards the production of cleft palate in this species.

Subproject B:

Effects of pancreatectomy in the Rhesus monkey.

146

Objectives:

This was a preliminary study to evaluate the effects of pancreatectomy in mature female Rhesus monkeys and to provide background information to enable evaluation of the effects on organogenesis at a later date. Two monkeys were initially used.

Major Findings:

A near total pancreatectomy (90%) was performed on the first monkey. This animal died within eight hours of surgery. The second monkey also received a 90% pancreatectomy. After an initial stormy post-operative course with wide variations in blood sugars from the low to high range requiring frequent monitoring and insulin. The animal gradually stabilized and over several weeks the insulin dosage was gradually decreased and later discontinued. After several weeks of no insulin therapy the animal was eating and reacting normally; blood studies and chemistries were generally normal except for a mild anemia and abnormal blood glucoses indicating definite diabetes. The blood sugars ranged near 100 in the morning and between 250 to 300 in the mid-afternoon (normal 75-100 during this time).

This preliminary study indicates not only the feasibility of using the subhuman primate as an experimental model for diabetes mellitus but also the potential use of these animals in sludying the effect of diabetes on growth and development.

Proposed Course of Project:

More pancreatectomies are anticipated in non-pregnant and later in pregnant and non-pregnant, but actively breeding, animals to further study the production of experimental diabetes in the monkey and its effects on growth and development.

Subproject C:

Aminopterin toxicity in the Rhesus monkey.

Objective:

To evaluate any adverse effect of aminopterin and to help determine a useful dosage for teratogenic studies in the non-human primate.

Methods Employed:

Two adult female Rhesus monkeys were used. One was given aminopterin orally by intubation at a dose level of 1 mg/kg and one at 10 mg/kg. Both were treated for five consecutive days. Observations

were recorded daily including body weights and pertinent clinical laboratory studies, including blood parameters, liver and renal functions, were carried out. Necropsy was conducted on deceased animals and on survivors at the termination of the experiment.

Major Findings:

The monkey treated with 10 mg/kg became ill on the final day of treatment, developed vomiting and weakness and died four days later. Four days prior to death the hematocrit white blood count was decreasing but still within normal limits. Blood chemistries were normal.

The second monkey was treated with 1 mg/kg for 5 days. This monkey did not become ill and all bleeding studies and chemistries remained within normal limits. On the basis of these studies it was elected to use doses of 0.25 to 1 mg/kg to begin teratogenic studies since this was approximately one tenth the lethal dose.

Proposed Course of Project:

Teratogenic studies using aminopterin will be carried out in pregnant monkeys and a critical dosage and time period established.

Part B: Not included.

Serial No. NIDR-28 (62)

1. Biochemistry
2. Cell Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Chromosome and Cell Growth Studies in Normal and Abnormal Subjects

Previous Serial Number: NIDR-89 (c)

Principal Investigator: Dr. H. L. Cooper

Other Investigators: Dr. J. J. Oppenheim, Dr. R. Stern, Dr. S. Handmaker, Dr. J. E. Kay, and Dr. J. Graef (Human Genetics Branch, NIDR).

Cooperating Units: National Cancer Institute, Dr. R. Friedman and Dr. B. Leventhal

Man Years:

Total:	10 1/2
Professional:	5 1/4
Other:	5 1/4

Project Description:

Objectives:

1. An intensive study of biochemical processes involved in the transition of non-growing human lymphocytes to a state of active growth leading to DNA replication and division. Special emphasis is placed on regulation of RNA and protein synthesis, and on ribosome function.

2. A study of the relationship of lymphocyte growth response in vitro to aspects of immunological phenomena in vivo.

3. A study of a hitherto undiscovered form of RNA found in animal cells, and its possible relevance to the question of the viral etiology of neoplasia or abnormal cell proliferation.

4. A study of the molecular organization of certain intermediate forms of viral RNA found during replication of an RNA virus in animal cells. (In collaboration with Dr. R. Friedman, NCI).

Methods Employed:

Lymphocytes for studies of ribonucleic acid metabolism were obtained from the peripheral blood of normal human volunteers. Lymphocytes were separated from the remainder of the blood cells by a differential adsorption technique.

The cells were stimulated to grow by addition either of phytohemagglutinin (an extract of the kidney bean) or of specific antigens. Metabolic changes were studied during the course of the subsequent cell enlargement and division. Radioactive precursors and metabolic inhibitors were used in various combinations to explore different aspects of cell growth stimulation. Ribonucleic acid synthesis in stimulated cells was studied by a variety of biochemical and physical means: RNA was extracted and purified by the phenol-sodium dodecyl sulfate method, its components separated by ultracentrifugation, and the behavior of individual components during different experiments was followed by assay of incorporation of radioactive precursor using liquid scintillation spectrometry. Components of RNA were also analyzed by their behavior during gel filtration and during chromatography on benzoylated DEAE cellulose columns.

Growth of lymphocytes in response to various agents was also measured by uptake of tritiated thymidine into DNA. A variety of agents known to stimulate lymphocyte growth, including purified phytohemagglutinins, specific antigens, anti-leukocyte antisera and antisera to specific cell products, are added to cultures under a variety of conditions, to investigate the dynamics of the lymphocyte response.

In some investigations, lymphocytes obtained from the lymph nodes of small mammals were employed.

Patient Material:

Lymphocytes for studies of normal cells were obtained from the blood of normal volunteers, collected in the Clinical Center Blood Bank by the usual procedures employed there. Nothing was done to any normal volunteer other than removal of blood. Blood specimens were also obtained from a variety of patients in the Clinical Center, admitted by other units and cared for by them. No drugs or other procedures beyond drawing of blood were used for our studies. No patient risk was incurred.

Major Findings:

1. a. A detailed study of the kinetics of the lymphocyte growth response to phytohemagglutinin (PHA) was completed. This

study used a combination of biochemical radioautographic and cytological techniques to determine the time-dose interrelationship between lymphocyte growth and optimal, sub-optimal and supra-optimal doses of PHA. It was shown that the time-course of stimulation of DNA synthesis was identical with different doses of PHA. Sub-optimal doses of PHA stimulated a smaller proportion of cells to respond, while supra-optimal doses stimulated a greater proportion of cells to respond, but also induced cell death. The overall effect of supra-optimal doses was to diminish DNA synthesis in the entire population because of reduced cell survival. Now under study is the question of whether the toxicity of supra-optimal doses of PHA is due to excessive activity of the same type as that involved in growth stimulation, or whether two simultaneous activities are present, affecting different aspects of cell metabolism. Dr. Handmaker was in charge of this investigation, in which Dr. B. Leventhal of NCI also collaborated.

b. In a study carried out by Dr. Kay, with Dr. Leventhal collaborating, the effects of inhibition of ribosome synthesis on protein and DNA synthesis by PHA-stimulated lymphocytes was examined. It was found that, when ribosome synthesis was abolished with low doses of Actinomycin-D, protein synthesis could still be enhanced by treatment with PHA. Protein synthesis increased until the point at which DNA synthesis usually begins in such cells, and these increased no further. Normal DNA synthesis did not begin. It was concluded that the ribosomes of resting lymphocytes are not functioning optimally in protein synthesis, and that their activity could be enhanced by PHA. It was suggested that the onset of DNA synthesis may be geared to the rate of ribosome synthesis or the number or density of cytoplasmic ribosomes. The onset of DNA synthesis may be further involved in the stimulation of synthesis of a variety of proteins.

c. Preliminary stages have been completed in establishing the methodology for a study of the RNA synthesis of isolated lymphocyte nuclei and their response to mitogenic agents either directly or as mediated through various subcellular fractions. This project is being carried out by Dr. Graef.

d. A detailed study has been completed of the early changes in the production of certain classes of RNA by lymphocytes as they shift from the resting to the growing state. Resting lymphocytes synthesize predominantly heterogeneous-sized RNA molecules which are rapidly degraded within the nucleus. Only 3-4% of the RNA synthesized during any short interval is stable ribosomal RNA. Upon treatment with the growth stimulant, PHA, there is an acceleration of all RNA synthesis, but ribosomal RNA synthesis increases disproportionately. After 24 hours of growth, overall RNA synthesis has increased over 20-fold, while ribosomal RNA

synthesis has increased 75 to 100-fold. This increase accounts for the marked accumulation of cytoplasmic ribosomes which distinguishes growing lymphocytes from resting ones. Several steps have been identified in RNA synthesis at which control mechanisms may exist which can be altered by a growth stimulant. These are:

i) Activity of pre-existing RNA polymerase. Pre-existing, apparently inactive, RNA polymerase can be activated to increase production of heterogeneous, but not of ribosomal RNA, without a requirement for new protein synthesis.

ii) Synthesis of large molecular precursor of ribosomal RNA (45s molecule). Continued synthesis of this molecule in resting lymphocytes requires continuous protein synthesis. Synthesis of ribosomal precursor RNA (unlike heterogeneous RNA) cannot be increased by PHA in the absence of protein synthesis. Thus, a critical requirement exists in the lymphocyte for a protein (or proteins) whose absence specifically prevents ribosomal RNA synthesis. This protein(s) may play a physiological role in cell growth regulation by fixing the rate at which ribosomal RNA may be produced.

iii) Conversion of ribosomal precursor RNA (45s) to an intermediate precursor molecule (32s). After addition of PHA, this conversion was shown to increase with different kinetics than the acceleration of 45s synthesis, and therefore may be subject to separate control.

This study was carried out by Dr. Cooper.

2. We are investigating the induction and immunological relevance of in vitro lymphocyte proliferation with antigen, nonspecific stimuli, and antisera in normal humans and other mammalian species. Variations from the normal in vitro response are found with lymphocytes from patients with immunological abnormalities such as Wiskott-Aldrich syndrome, ataxia-telangiectasia, aphthous stomatitis and hypogammaglobulinemia.

We are studying the intercellular relationship of macrophages and lymphocytes which we have found promote lymphocyte proliferation in vitro. We have also found that macromolecular polycations block lymphocytes in vitro. This block can be overcome by polyanions, which at times can enhance the lymphocyte growth. The effects of these synthetic polymers parallel those of soluble anti-immunoglobulin:immunoglobulin complexes which have a nontoxic inhibitory effect on the lymphocytes in vitro and may reflect a feedback control mechanism of in vivo lymphocyte response to antigenic stimuli. This work is being carried out by Dr. Oppenheim.

3. In order to improve our ability to separate and study various types of RNA produced by the cell, a chromatographic technique was developed by Dr. Stern, employing benzoylated DEAE cellulose and a variety of eluting gradients. RNA molecules are distinguished by their degrees of secondary structure. Using this technique, an unusual form of RNA was isolated from cultured Burkitt lymphoma cells which has characteristics resembling those of a viral intermediary form. This material is now under intensive study.

4. In collaboration with Dr. R. Friedman, NCI, Dr. Stern has verified the multi-stranded nature of the viral replicative intermediate form of Semliki-forest virus. Replication of the nucleic acid of this RNA virus appears to take place on a double stranded RNA template, analogous to the normal transcription of RNA on double stranded DNA in the cell nucleus.

Significance to Dental Research:

The studies performed in this project are aimed at an understanding of the mechanisms by which genetic information contained within the cell functions in the regulation of normal and abnormal cell growth and cell differentiation. Such knowledge is of basic importance in the dental and medical fields, where disordered cellular differentiation may result in congenital malformations, and where control of cell growth may become disordered with resultant neoplasia.

Major problems to be surmounted in this field include those preventing successful transplantation of teeth and progress in the field of histocompatibility typing would certainly be a major step to achieving this goal.

There are some oral cavity disorders which are related to contact hypersensitivity, "autoimmunity" and allergic reactions. Elucidation of these cellular responses involved may lead to improved therapeutic approaches for these conditions.

Proposed Course of Project:

Research will continue primarily in the area of cell growth regulation, as studied in peripheral blood lymphocytes which may be stimulated to shift from a resting state to active growth. It is hoped that the mechanisms concerned with maintaining the resting state, and the alterations which trigger the onset of cell growth will be further elucidated.

An understanding of the cellular mechanisms which regulate the synthesis of messenger RNA is another area of study to be pursued.

This information will be important for an understanding, not only of control of cell growth, but also of cellular differentiation. The interaction between lymphocytes and macrophages will be studied to learn how the cell types communicate, and how this may be achieved synthetically. Increasing emphasis will be placed on phenomena occurring at the cell surface during growth stimulation, as part of the continuing effort to elucidate the initiating events in the onset of cell growth.

Unusual RNA forms found in normal and malignant cells will be studied in greater detail in an effort to understand their role in cell function.

Part B Publications:

Friedman, R., and Cooper, H.: Stimulation of interferon production in human lymphocytes by mitogens. Proc. Soc. Exp. Biol. and Med. 125: 901-905, July 1967.

Cooper, H. L.: Studies on early biochemical changes in phytohemagglutinin-stimulated lymphocytes. In Rieke, W. O. (Ed.): Proc. 3rd Annual Conference on Leukocyte Culture. Appleton-Century-Crofts. In press, 1968.

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Bradley, J., and Oppenheim, J. J.: The in vitro proliferation of lymphocytes from patients with hypogammaglobulinaemia. Clin. and Exper. Immunology 2: 549-557, 1967.

Oppenheim, J. J.: The relationship of in vitro lymphocyte transformation to delayed hypersensitivity in guinea pigs and man. Fed. Proc. 27: 21-28, Jan-Feb. 1968.

Leventhal, B. G., and Oppenheim, J. J.: Effect of cell density on the rate and degree of response of leukocyte and purified lymphocyte cultures. In Rieke, W. O. (Ed.): Proc. 3rd Annual Conference on Leukocyte Culture. Appleton-Century-Crofts. In press, 1968.

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Knight, S., Bradley, J., Oppenheim, J. J., and Ling, N. R.: The *in vitro* proliferation of human thymoma, normal rabbit and guinea pig thymocytes. Clin. and Exper. Immunol. In press.

Oppenheim, J. J., Leventhal, B., and Hersh, E. M.: The transformation of column purified lymphocytes with nonspecific and specific antigenic stimuli. J. Immun. In press.

Handmaker, S. D., Leventhal, B. G., and Cooper, H. L.: The kinetics of PHA-stimulation of human lymphocytes. In Rieke, W. O. (Ed.): Proc. 3rd Annual Conference on Leukocyte Culture. Appleton-Century-Crofts. In press, 1968.

Kanfer, J. N., Richards, R., Kampine, J. P., Handmaker, S., and Yankee, R. A.: Alteration of the sphingolipid content in leukocytes from patients with Chediak-Higashi syndrome. Life Sci. 6: 2661-2664, 1967.

Annual Report of the Human Genetics Branch
National Institute of Dental Research
Summary Statement

The Human Genetics Branch has been divided into two sections which cover the two major areas of research being done in the Branch. The Population Genetics Section carries out field studies on the variations and defects of oral structures in human populations and the relationship to the genetic structure and mating systems of different population groups. The Developmental Genetics Section uses a variety of laboratory methods to study the genetic mechanisms controlling the development of the structure and function, and to study the effects of genes involved in developmental defects of laboratory animals. The functions of the two sections compliment each other in the combination of laboratory and population techniques used to study specific defects, such as cleft palate, and specific genes such as those causing Waardenburg Syndrome in both men and animals.

MALOCCLUSION

Two studies are being conducted in this area; the first is a study of racial variation with emphasis on the effects of racial crossing. This study is being conducted in collaboration with the School of Public Health at the University of Hawaii. The past year has been spent almost entirely in data collection, with examinations completed on approximately 19,000 high school students. Other studies of malocclusion are being conducted in the Papago Indians. Although the results are quite preliminary, comparison of present Papagos with ancestral skeletal remains suggest a marked increase in the frequency of malocclusion in the period since Spanish contact. It is believed that these dental changes are the result of changing culture and dietary patterns rather than of genetic origin.

CLEFT LIP AND CLEFT PALATE

Continued utilization is being made of data collected in past studies. A significant finding has been the failure to confirm previous reports of minor anomalies in clinically normal relatives of oral cleft patients using laminographic x-ray procedures. Previous studies have been without benefit of controls; hence, the present results are considered more reliable. Another finding has been the demonstration of mid-facial changes (relative hypertelorism) in patients with cleft lip. Normal relatives of these patients do not show the same changes, suggesting that this is a phenomenon secondary to the cleft lesion itself, rather than a basic genetic morphological or growth difference. Collaborative arrangements have been made for the use of two large bodies of data on oral clefts. These are being utilized for studies of genetic segregation and for analysis of geographic and temporal clustering.

AMERICAN INDIAN CONGENITAL MALFORMATION

To date approximately 28,000 consecutive newborn records have been obtained from the Division of Indian Health hospitals. Significant has been the finding that for certain malformations (cleft lip and spina bifida) the American Indian shows frequencies intermediate between Caucasian and other Mongoloid populations. For other defects (isolated cleft palate and anencephaly) Indians still retain frequencies quite similar to other Mongoloids. Indian newborn data are also being used for the studies of birth weight. The most significant result is the development of a mathematical model, which shows promise of considerable utility in epidemiological studies of prenatal factors affecting birth weight.

POPULATION GENETICS

These studies include theoretical approaches involving the development of mathematical models and computer simulation as well as collection and analysis of data from human populations. Methods are being developed for the simultaneous analysis of two or more genetic loci. Human data is invariably collected in this form but is rarely analysed for more than one locus at a time. A multi-locus approach should provide a great deal more information on population variation than consideration of loci individually.

STUDIES OF HUMAN CHILDHOOD DEAFNESS

The study of the medical histories of the past and present pupils of the Clarke School for the Deaf has shown that the major exogenous causes of deafness in early childhood are infection of the central nervous system in infancy or in utero (rubella), and severe prematurity with birth weights under four pounds. These conditions account for less than 15% of childhood deafness. About three-fourths of all childhood deaf do not have any history of disease in the newborn or prenatal periods. Statistical and genetic data suggests that about 40% of childhood deafness results from simple recessive genes and 15% from dominant genes. The remaining 20% are undifferentiated, but may be either of complex genetic origin or the result of undiagnosed diseases.

SALIVA STUDY

The etiology of polymorphism in the recently discovered isoamylases of human parotid is under continued study. Family populations including twins are being utilized to discover genetic control. Isolated disease states are being analysed for possible environmental effects. A method for the study of isoenzymes of lysozyme as well as amylase is available for serum, urine and saliva.

A small molecular weight protein-like substance has also been identified. This was found to be more prevelant in parotid saliva of those persons having high rates of calculus formation. Further studies of this substance are in progress.

STUDIES OF TASTE AND SMELL

Variations in the ability to taste specific chemical compounds, such as phenylthiourea and anetholetrithione, have been shown to be under genetic control. The classical view that the various taste receptors are restricted to the tongue has recently been abandoned, since the palate and pharynx are now known to possess taste receptors. Their stimulation appears to occur consequent to a loose coupling of the effector substance with proteins in the taste buds.

Preliminary studies of twins have shown that identical twins are much more alike in taste responses than are like-sex fraternal twins, but that the relationship of a olfactory response is more complex. A specific chemical odor perception defect for isovaleric acid is being analyzed by family study for mode of inheritance, while other analogous odor blindnesses are being sought by screening populations.

EXPERIMENTAL STUDIES OF GENETICALLY CONTROLLED DEVELOPMENTAL MALFORMATIONS

The control of the defect of hair formation in the downless mouse is being studied in a tissue culture system, in which the effects of the mesoderm as an inducer of differentiation of ectoderm will be determined in normal and genetically defective mice. The downless state is of interest because it appears histologically to result from a failure in the initiation of hair follicle formation. Since the teeth develop as downgrowths into underlying mesoderm as do hair follicles, follicular growth may provide a convenient analog of tooth development. A specific mutant in the mouse, whose genetic defects include amelogenesis imperfecta and abnormal hair development, has also been identified. This mutant is now also being studied both genetically and histologically.

The hereditary defects of the domestic cat produced by a single dominant gene have been studied in detail. Defects include hearing loss, as well as ocular and pigmentary anomalies. Hearing loss varies in degree and may be unilateral or bilateral. This is apprently secondary to a failure in the last stages of maturation of the cochlea, particularly in the production of endolymph, and is manifested by varing degrees of collapse of the membranous labyrinth. Depending on the localization of the collapse the hearing deficit is variable. The defect in the formation of the eye of these cats is independent of the ear anomaly. Congenital absence of the tapetum, when it occurs, is complete within an affected eye. Even when the mutant gene is present, however, the tapetal anomaly may occur in both, one or neither eye.

Serial No. NIDR-29 (62)
1. Human Genetics
2. Developmental Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Discrimination and Segregation Analysis of Hereditary Deafness in the Students of the Clarke School for the Deaf.

Previous Serial Number: NIDR-86 (c)

Principal Investigator: Dr. K. S. Brown

Other Investigators: None

Cooperating Units: The Clarke School for the Deaf, Northampton, Massachusetts; National Institute of Neurological Diseases and Blindness; School of Public Health, University of Hawaii.

Man Years:

Total:	1
Professional:	1/4
Other:	3/4

Project Description:

Objectives:

1. To discriminate the various types of deaf people into groups based on the characteristics of their conditions, including patterns of audiogram, physical findings and laboratory values.

2. To analyze the patterns of genetic segregation among the various types of hereditary deafness in order to estimate the number and kind of genetic factors causing deafness.

Methods Employed:

Personal examination of about 300 deaf people and 700 of their hearing relatives, including parents, grandparents, and siblings, has been completed. The examination has been limited to the head and neck, except for blood pressure and cardiac auscultation. It has included caloric and turning-chair vestibular examinations, oral examination with DMF, otoscopic and ophthalmoscopic

examinations of 170 deaf children who are currently attending the Clarke School, and a similar examination lacking the caloric and dental examinations, but including a pure tone audiogram for the hearing relatives. All but the youngest children were given a PTC taste test, a routine microscopic urinalysis, and urine analysis for glucose, protein, and phenylketone-like substances.

Blood samples were drawn for genotyping and laboratory studies of the serum. These include protein and lipoprotein electrophoresis, total protein, total lipid, and cholesterol determinations. All individuals showing clinical goiter, together with their sibs and parents, have been examined for protein bound iodine. A contract has been completed by Bionetics Research Laboratories, Inc., under NINDB support to carry out the serum studies exclusive of the electrophoresis.

The pedigree and history material collected by the Clarke School has been examined, and a questionnaire form to supplement and update it has been prepared (Budget Bureau No. 68-6229), and over 2,000 copies have been distributed. The resultant data are being coded by employees of the Clarke School under NIDR direction on two computer coding sheets prepared for this purpose (PHS-T-96, PHS-T-97). This work is being done under a contract between the Clarke School and NINDB. The resulting coded data is being processed at the NIH computer center, utilizing the SEGRAN program, developed by Dr. Chung.

Patient Material:

Patient material consists of the students at the Clarke School for the Deaf and their siblings and parents or other relatives, if indicated. The alumni of the Clarke School and their families, where possible, will be examined, including as many of the graduates since 1930 as are still available. An estimated 1,500 people are available of whom about 700 are deaf.

Major Findings:

Genetic information has been coded on 5,553 persons related to Clarke School pupils of whom, 1,471 are deaf. This included over 99% of pupils attending Clarke School since 1930, 96% of those attending since 1920 and 47% of all pupils who have ever attended Clarke School. The processing of the records of the genetic data has been completed and the analysis is in progress at the computer center, School of Public Health, University of Hawaii and in the Human Genetics Branch, NIDR.

Evaluation of the medical records and physical findings on the present and former pupils of Clarke School shows that about 20% of the childhood deaf were the result of classifiable disease,

accident or toxic condition. An additional 4% resulted from unclassifiable, but probably extrinsic causes, while 76% were the result of hereditary factors or undetected disease. Genetic analysis further suggests that undetected disease accounts for 18% of the total, so that 58% of the defects observed have a simple hereditary basis.

Among the acquired cases, the major cause of deafness (32%) is acute CNS disease, such as meningitis or encephalitis of which measles is the most common cause.

The second most common cause of extrinsic childhood deafness (26%) is undifferentiated severe prematurity with birth weights under 5 1/2 pounds. Most of these are severe prematurity under 4 pounds at birth but there is a less severe group that is possibly confounded with prenatal rubella which was not diagnosed.

Gregg's syndrome due to prenatal rubella is the third most common extrinsic cause of deafness and accounts for 14% of the classifiable cases.

Other prominant extrinsic causes, each producing just over 5% of the total classifiable cases, are suppurative otitis, neonatal jaundice, and acute febrile illness without CNS history.

Genetic analysis shows that simple recessive mendelian factors account for about 40% of childhood deafness. Several methods suggest that there may be between 50 and 100 individual genes involved, although there may be more very rare genes that also cause deafness. About 15% of deafness is due to dominant genes, also of many types. The selective disadvantage of deafness, suggests the probability that recurrent mutation is the source of genes causing deafness.

Laboratory study of the genetics, physiology, and anatomy of the congenital hearing loss that may occur in the domestic cat is being carried out as an extension of our interest in the Waardenburg's syndrome of man. Affected cats, like Waardenburg's syndrome people, show variable degrees of hearing loss and lack of normal pigment in the hair and eye. A colony of these animals is being bred for study of the physiological and anatomical characteristics associated with varying degrees of handicap.

Significance to Dental Research:

Development of diagnostic criteria which allow the separation of various groups of congenital and non-congenital deafness is basic to the understanding of possible etiologic factors involved.

Development of genetic information about a complex congenital malformation of known genetic origin may have a guiding role in the analysis of the complex genetic factors in such oral malformations as cleft palate and malocclusion.

Proposed Course of Project:

The data collection phase of the study of the Clarke School pupils and relatives has been completed. The statistical and genetical studies on the data are in progress and this work will continue for at least one additional year. The analysis of the segregation patterns will be the basis for genetic conclusions regarding the quality and quantity of simple genetic factors involved in the production of childhood and adult deafness. Further detailed examination of those family records in which there is interaction of environment and genetic factors will be undertaken.

The study of anatomical and physiological characters of the white cat with hearing loss will be continued. The electrophysiology of the cochlea in these defective animals will be evaluated in relation to normal controls. The genetics of the defect will be studied by continued breeding experiments.

Part B

Publications:

Brown, K. S., Hopkins, M. S., and Hudgins, R. B.: Causes of childhood deafness. In: Proceedings of International Conference on Oral Education of the Deaf -- (Ed. 1), by the Alexander Graham Bell Assoc. for the Deaf, Inc., 1967, Vol. 1, pp. 77-107.

Serial No. NIDR-30 (54)
1. Human Genetics
2. Developmental Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Environment and Genetic Factors in Taste and Smell Abilities

Previous Serial Number: NIDR-88 (c)

Principal Investigator: Dr. K. S. Brown

Other Investigators: Mr. C. J. MacLean, Mr. W. C. Leyshon

Cooperating Units: None

Man Years:

Total:	1 1/4
Professional:	3/4
Other:	1/2

Project Description:

Objectives:

1. To describe variation between individuals in the ability to detect the odors and taste of specific chemical compounds.

2. To evaluate the nature of the relationships between the thresholds for taste and smell of different compounds in the same individual.

3. To evaluate the genetic and non-genetic contributions to the absolute threshold for specific compounds.

4. To evaluate the genetic and non-genetic contributions to the relationships between the thresholds for different compounds in the same person.

Methods Employed:

Serial dilutions, in order of increasing concentration of the specific compounds, are presented to the subjects who state whether they can or cannot detect any odor or taste. The level of first detection is the threshold which is used as the data base for analysis.

The subjects include:

1. Sixty young adults without known illness for a study of the interactions of eight odors.

2. Two thousand school children for a study of six odors and the ability to taste phenylthiocarbamide (PTC).

3. Eighty pairs of parents and their children in a study of two odors and ability to taste PTC.

4. One hundred and fifty students in a study of the correlation between ability to taste PTC and the ability to taste anetholetrithione.

5. Ten pairs of same sex twins in a study of PTC, anetholetrithione (ATTH), and isobutyric acid.

Major Findings:

The threshold for the ability to smell most chemical compounds is controlled by general sensitivity factor and by special factors related to the specific chemical properties of the particular odorants. Preliminary evidence has identified two of these factors as being related to the capacity of the odorant to undergo molecular ionization as an acid or as a base. A third factor is related to molecular size or weight.

The threshold for the smell of cyanide ion appears to be controlled by different factors, and the smell may be related to metabolic properties of cyanide rather than to its molecular properties as an odorant in the usual sense.

Family studies of the threshold for the odor of cyanide have not supported the published claims that the threshold varies as a simple genetic trait.

The ability to taste PTC has been long recognized as a simple genetic trait. ATTH is a compound of very different molecular structure, which has been reported to have a taste threshold inherited as a genetic trait independent of PTC. Our study showed that there is a high correlation between the ability to taste these two compounds.

Twin studies are not conclusive, because of small numbers, but they suggest that PTC and ATTH are genetically independent.

Significance to Dental Research:

The senses of taste and smell are two of the least known senses. The mechanisms involved are unknown and only a few studies of genetic

aspects of those abilities have been attempted. Application of genetic and statistical techniques to quantitative threshold data on these abilities may produce increased understanding of these two special senses involving the oral cavity.

Proposed Course of Project:

Further study of data already collected may suggest direction for new experiments. The immediate goal is to further evaluate the nature of the factors controlling receptor thresholds. This will involve tests on a series of subjects with a wide variety of compounds showing relationship to those already tested.

Search for odorants showing specific variations in different individuals will also be continued. These will be analyzed by both family and twin study techniques.

Part B

Publications:

Brown, K. S. and Robinette, R. R.: No simple pattern of inheritance in ability to smell solutions of cyanide. Nature 215 (5099): 406-408, July 1967.

Serial No. NIDR-31 (63)
1. Human Genetics
2. Population Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Genetic Studies of Oral Diseases, Anomalies and Development

Previous Serial Number: NIDR-90 (c)

Principal Investigator: Dr. D. W. Runck
Dr. J. D. Niswander

Other Investigators: Mr. C. J. MacLean

Cooperating Units: School of Public Health, University of Hawaii

Man Years:

Total:	3
Professional:	2
Other:	1

Project Description:

Objectives:

1. The purpose of the study is to clarify the relative role of genetic and environmental factors in certain oral conditions.

   a. To determine the effects of inbreeding and hybridization on malocclusion and dental caries. Specifically; do we obtain an F_1 effect in the frequencies of these conditions, is there evidence of a recombination effect on dental characteristics.

   b. To assess the role of environment in malocclusion--specifically has the frequency, type and severity of malocclusion changed over time, and if so, can the changes be related to socioeconomic status and changing cultural patterns.

   c. To assess the correlation between relatives in malocclusion.

   d. Elucidate genetic mechanisms involved in morphological variations of the teeth.

2. Utilize the teeth and oral cavity in the study of basic genetic phenomena particularly in the field of quantitative genetics. It is relatively easy to obtain large amounts of data, particularly in family groupings. Also, good methods exist for permanent capture of the raw data (casts and X-rays). Therefore, the teeth and oral cavity offer an excellent area for the study of biometrical genetics.

Methods Employed:

Effort during the last year has been focused in two areas: (1) survey of oral conditions of high school children in Hawaii, and (2) studies of malocclusion in the Papago Indians.

The Hawaiian project is designed to study the causes of racial variation in dental characteristics, particularly malocclusion, with emphasis on the effects of racial crossing. The past year has been spent in data collection which is expected to be completed in the summer of 1968. At that time approximately 20,000 individuals will have been examined, the data processed and ready for analysis.

This study concerns itself primarily with a population sample ranging in age from 12 to 17, i.e. individuals born between 1949 and 1954. Reasons for selecting this group of children are as follows: Firstly, the individuals of this age group are in the intermediate schools (grades 7, 8, and 9) or the high schools (grades 10, 11, and 12) so that examination of subjects can be done readily through the public school systems. Secondly, this age group has well-developed permanent dentition, as well as readily discernible malocclusion, when this defect is present.

The examination includes the following items: (1) evaluation of occlusions based on the Angle classification, and on other criteria by visual inspection; (2) simple functional evaluation of malocclusion; (3) intra-oral measurement of mesio-distal diameter of the upper central incisors; (4) the number of teeth, noting congenitally missing teeth; (5) scoring of periodontal disease based on Russell's method (1965); (6) status of oral hygiene; (7) scoring of dental caries (DMFS, DMF); (8) presence or absence of cleft palate and/or harelip; (9) presence or absence of other oral anomalies, and (10) anthropometrics including height, weight, and cephalic measurements.

Automated linkage of recorded data will include coupling of preexisting information on the birth certificates (already on tape) with the dental characteristics obtained by the present examinations. In progress is an extensive genetic study with record linkage by Drs. Mi and Morton of the Department of Genetics of the University of Hawaii. They are collecting biological, medical and sociological information on all individuals involved in marriage, birth, and death in Hawaii since 1942. This enormous file will be a great

potential source of genetic and epidemiological studies of medical and dental health.

Additionally, since 1930, the Strong-Carter Dental Clinic of Honolulu has been offering annual free dental service to over 4,000 children of "indigent" families. The eligibility requirement for enrollment with the Clinic is such that 17% of the students in the public schools are in this category at the present time. As each child registers with the Clinic, he is given a general dental examination, including posterior radiographs. Information on race, sex, birth date, general health condition, and sociological status is also collected. Thereafter, until age 12, regular annual examination and treatment continue. Thus, these data, though biased with respect to the socioeconomic status of the family, will supplement the first source of data in the studies of tooth abnormalities, particularly as regards missing teeth and dental caries. These records will be linked by the computer with other sources of data to supplement epidemiological information and to eliminate duplications. The obstetric and pediatric information from the Kapiolani Maternity Hospital will also be used in the record linkage work.

The Papago studies have involved the field examination of approximately 300 school children and the collection of dental casts on about 140. Primary concern is the evaluation of tooth alignment and occlusion. An attempt is being made to collect similar data from skeletal remains of ancestors of the present Papagos. Suitable skeletal material is quite scarce, with only about a dozen specimens examined to date. Arrangements have been made to accompany archeologists from the Amerind Foundation on their next excavation. It is hoped that considerably more material will be obtained through this source.

Serological genotypes are being determined for the purpose of defining biological subgroups of the Papago. Approximately 15 red cell and serum markers are being evaluated. Certain oral conditions (primarily occlusion and dental morphology) are being evaluated simultaneously. If clear-cut genetic differences within the tribe can be established as a consequence of the blood studies, these genetic subgroups can then form the basis for study of subtle differences in dental and oral conditions.

Major Findings:

The studies in Hawaii have to date involved only data collection. There are therefore no major findings to report. Frequencies of the four major classes of malocclusion, based on molar relationship, are shown below for the Papago and other groups examined by identical methods.

	Papago Indians	Bakari Indians	Xavante Indians	Japanese	Caucasian
No mal-occlusion	.33	.55	.95	.41	.36
Class I	.48	.31	.05	.44	.30
Class II	.14	.07	---	.12	.24
Class III	.05	.07	---	.03	.10

The Xavante represent an essentially untouched tribe in central Brazil, whereas the Bakari, although presently residing in close proximity to the Xavante, have been in permanent contact with modern Brazilians for over 50 years, and have undergone considerable acculturation. Among the Indian groups there is a striking association between the degree of acculturation and the frequency as well as severity of malocclusion, the Papago data closely approximating those for modern Japanese. To date only 10-12 intact early Papago dentitions have been examined. These date roughly from a period between 1300 and 1700 AD. All have shown nearly perfect occlusion!

These findings, together with previous results, suggest that while recessive genes play a role in the development of malocclusion, environmental factors are probably of most importance. Studies of siblings suggest that these environmental factors are correlated within families, and genetic factors are probably more of a predisposing than causative nature.

Although these data do not allow very definitive conclusions by themselves, they are consistent with the other results and add considerable strength to the interpretation that environmental factors are of great importance in the etiology of malocclusion.

Significance to Dental Research:

A great deal of speculation presently exists as to whether the high frequency of malocclusion in civilized man represents the untoward results of changing diet (either nutritional or functional), the results of evolution (relaxation of natural selection against occlusal disharmonies), or the result of admixture of diverse physical types. The majority of evidence seems to indicate the fact that primitive populations in general are characterized by lower frequency of malocclusion. There is also evidence to suggest the same trend applies to periodontal disease.

The present studies are designed to yield information on these questions and are, therefore, of significance for understanding the etiology of oral developmental anomalies and malocclusion.

Such knowledge should have eventual application in prevention and treatment.

Proposed Course of Project:

To continue collecting data on "primitives" as opportunities are available and to further search out and examine groups for which skeletal material exists, and otherwise fulfill sufficiently the requirements outlined at the beginning, and to collect data on oral conditions in living North American Indian groups with particular reference to malocclusion. The Papago Indians of Arizona appear to fulfill many of these requirements. It is planned to continue collecting data on this group.

To proceed with analysis of the data presently being collected in Hawaii.

Part B

Publications:

Bailit, H. L., Thompson, L. A., and Niswander, J. D.: Dental eruption and hypodontia. J. Dent. Res., July 1968 (in press).

Serial No. NIDR-32
1. Human Genetics
2. Population Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Theoretical and Applied Analyses in Human Populations with Particular Emphasis on the Study of Genetic Variation.

Previous Serial Number: None

Principal Investigator: Dr. P. L. Workman

Other Investigators: Mr. C. J. MacLean, Mr. W. C. Leyshon

Cooperating Units: None

Man Years:

Total:	2 1/2
Professional:	1 1/4
Other:	1 1/4

Project Description:

Objectives:

1. To determine which particular factors are influencing the pattern of genetic variation in any population at a given time. Of especial interest are such factors as assortative mating, selection, population intermixture, and population size.

2. To develop suitable methods for the analysis of intra vs inter population differences. Particularly important are those techniques which permit an assessment of the relative stability of genotypic distributions over space and time. This work will concentrate on the differences within and between North and South American Indian populations.

3. To assess which indicators of developmental instability in humans are useful for comparing populations under genetic stress (severe inbreeding for example) or environmental stress (poor nutrition, high disease rates). Two particular measures of instability are being analyzed in different populations: (1) the asymmetry of fingerprint ridge count, and (2) the

asymmetry with respect to measurement characters of homologous teeth.

4. A theoretical analysis of the manner in which dominance variation has evolved, with special reference to the evolutionary origin of stable buffered developmental pathways.

Methods:

Methodology in this area involves both data analysis and more theoretical mathematical procedures, ranging from model building to computer simulation. The major source of data presently being used is the genetic information obtained from field studies of the Papago Indians in conjunction with the social demographic and genealogic information available in the Papago tribal register.

Major Findings:

1. The determination of the appropriate methods for separating the effects of intermixture between populations from changes in the genetic structure due to selection has shown that for several human polymorphisms, in particular populations, selective forces can be demonstrated. Since the forces are generally not large, this method, aimed at assessing the cumulative effects of the separate forces over a period of several generations, may be extremely useful for detecting the existence of selective mechanisms.

2. The analysis of human population data in terms of two or more loci, considered simultaneously, is shown to provide far more information on the long term pattern of variation in a population than that normally obtained by a consideration of one locus at a time. Further evidence is provided to show that single-locus analyses only reveal the most extreme imbalances in population structure.

Significance to Dental Research:

In addition to developing the theoretical framework for population analysis, the results should indicate which populations might be most profitably studied in great detail, particularly with respect to demonstration of selective forces, and determination of the biological forces underlying them.

Proposed Course of Project:

Continuation of same program for another year.

Part B

Publications:

Workman, P. L.: Gene Flow and the Search for Natural Selection in Man. Human Biology. May, 1968 (in press).

Serial No. NIDR-33
1. Human Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Studies on Site of Action of Phytohemagglutinin on Circulating Human Lymphocytes

Previous Serial Number: None

Principal Investigator: Dr. J. W. Graef

Other Investigators: Dr. H. L. Cooper, Laboratory of Biochemisty

Cooperating Units: None

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

1. To determine those portions of the cell required for in vitro activation of RNA polymerase in human lymphocytes.

2. To elucidate the mechanism of stimulation of the human lymphocyte by phytohemagglutinin (PHA).

Methods Employed:

Blood is drawn from normal, healthy volunteer donors and the lymphocytes purified by standard techniques. These are placed in culture in vitro for varying lengths of time from 16-48 hours and are harvested. Cells are disrupted and preparations of nuclei are obtained with an average purity of 70-80 percent. These are incubated in the presence of labeled RNA precursors and necessary cofactors and uptake of label is considered a measure of activation of the RNA polymerase enzyme reflecting the potential "transforming" and growth activity of the particular culture. The nuclei are incubated with and without phytohemagglutinin and various cell fractions are added to the system both from cells previously exposed to phytohemagglutinin and from resting cells to determine those fractions most greatly enhancing phytohemagglutinin activity.

Patient Material:

Normal volunteers.

Major Findings:

Nuclear preparations of 70-80 percent purity, in the absence of cytoplasmic additions are not stimulated by phytohemagglutinin. If anything, phytohemagglutinin appears to interfere with the uptake of labeled RNA precursors in the resting nuclei.

Significance to Dental Research:

The phytohemagglutinin mitogen offers an experimental model for the study of cell division and the basic events involved in the transition of cells from resting to active. Because the human lymphocyte's specialized functions effect a wide range of human disease and, in general, are involved to some extent in virtually every inflammatory condition known, including periodontitis, aphthous stomatitis and resistance to herpetic infection, elucidation of mechanisms whereby this cell is stimulated is of fundamental importance. It also offers a model for the study of similar activity in other cell types not so easily obtained in vitro.

Proposed Course:

The initial experiments involving the bio-assay for nuclear activity and base-line measurements of this activity, both with and without PHA, have been completed with what appears to be reliable, reproducible data. It is proposed to move on to the major portion of the project involving measurement of the effect of different cell fragments on the nuclear system. Problems anticipated are technical and involve the difficulty of preparing biologically active fragments in amount and concentration necessary to effect the bio-assay system.

Part B not included.

Serial No. NIDR-34
1. Human Genetics
2. Developmental Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Developmental Processes in Genetically Controlled Traits

Previous Serial Number: None

Principal Investigator: Dr. K. S. Brown

Other Investigators: Dr. D. R. Bergsma, Dr. J. A. Sofaer

Cooperating Units: Dr. E. L. Eagles, NINDB

Man Years:

Total:	2
Professional:	1 1/2
Other:	1/2

Project Description:

Objectives:

1. To describe the development of experimental animals with genetically produced congenital malformations, particularly those of dentition and the oral region.

2. To examine the interaction between the tissues of these animals in the developmental processes in order to determine the nature of the mechanism producing the genetic defect.

Methods Employed:

1. A survey of mouse mutants is being undertaken to evaluate their oral structures in relation to those of the lines from which they arose.

2. Mutants of specific interest are being collected, and colonies of these animals are to be developed as a basis for experimental study.

3. Timed matings are being made to produce animals of known gestational age. Serial sacrifice and histological study will

produce systematic documentation of the developmental processes.

4. Organ culture is being undertaken of genetically defective skin and related tissues. Comparative studies of the development of mutant ectoderm with normal mesoderm and vise versa are being made to assess the nature of the developmental defect.

5. In the cat, the dominant genes producing defects of pigmentation, hearing and eye color are being studied in a colony of 80 defective animals being maintained by NINDB. Histological and physiological studies of these animals are being made. Studies of the function and survival of these animals in nature as compared with the colony are also being made in collaboration with the Animal Quarantine Station at Poolesville.

Major Findings:

Two mutants showing dental defects have been identified and detailed studies are underway. One of these occurs in the mutant (dn) downless, in which both hair and tooth development are defective in their initiation. Another mutant exhibits recurrent alopecia, and appears to have an amelogenesis imperfecta-like dental condition. Several other mutants have been examined and rejected for further study.

The mutant downless tissues have been chosen for the first organ culture studies, and cultures of separated ectoderm from early embryonic developmental stages have been made. These cultures have demonstrated the ability to separate the germ layers and maintain growth. Experimental cultures are just being started. They will attempt to localize the site of the specific defect and also to see if the site changes with the stage of development of the tissue.

Studies of fetal histologic material from kittens with the dominant gene or genes producing the syndrome of deafness, white fur and heterochromia, or bilateral blue iris, have been carried out using serial section mapping of the membranous part of the inner ear. The anatomic defect does not appear in the stages before birth, but is only visible histologically in the early neonatal period, when the inner ear is undergoing its last differentiation and beginning to function. Correlated with these changes in the normal kitten are changes of the sulcus cells of the organ of Corti. In the abnormal animals, there appears to be a lack of production or regression in the production of endolymph. The Reisners membrane is partly or completely collapsed onto the organ of Corti. Hyalinized bodies occur in the stria vascularis in older animals. A comparable finding has been reported in viral disease, but its occurrence in genetic disease suggests that it is a common degenerative result rather than specific to any one etiology.

Studies of the histology of the iris and retina in genetically defective white cats suggest that there is an absence of certain differentiating stimuli. The anterior layer of the iris, which normally contains melanocytes, is not pigmented. The tapetum, a special structure occurring in the pigment cell layer of the retina is not developed although some pigment is formed. The rest of the retina is physiologically and histologically normal. Because the tapetal defect can occur unilaterally, the opportunity exists to test several theories of its function. Genetic studies have shown that the eye and ear defects are not localized to the same side on any one animal. They are probably examples of the complete penetrance of a pleotropic gene or gene complex. Breeding experiments are underway to determine whether one or many genes are involved in this trait.

Significance to Dental Research:

Early development of teeth and hair both result from the down-growth of localized ectoderm into underlying mesoderm. The analysis of the interactions of ectoderm and mesoderm is fundamental to the understanding of the development of teeth. The use of animals in which specific genes produce defects in the normal processes will allow analysis of the mechanism of interaction and its genetic control.

Neural crest cells are a part of the early ectoderm that gives rise to pigmentation and to the sensory neurones. The generalized but incomplete expression of the anatomic defect in the cochlea of the "deaf white cat" suggests that the syndrome is a result of a defect in the embryology of the neural crest cells. If the hypothesis of a neural crest defect can be tested, it may have implications for the development of the facial complex in Waardenburg's syndrome of man, a human analog or homolog of the cat syndrome. The genetics of the cat syndrome may also suggest the nature of the genetics in man.

Proposed Course of Project:

Further surveys of mutant mouse lines with respect to dental defects will be undertaken. Colonies of specific mutants will be established and culture studies of the development of the defect undertaken. Genetic, embryological and histological approaches to the mechanism of genetic defects will be undertaken along the same lines already in progress.

Part B

Publications:

Brown, K. S., Wakeford, O. S., and Binder, P. A.: Knife-wetting device; wet celloidin technique. Arch. Otolaryn. 87: 131, April 1968.

Serial No. NIDR-35 (66)
1. Human Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: A Study of the Relationships between Genetic Factors, Exposure to Vitamin D *in utero*, and Buphthalmos in the Rabbit.

Previous Serial Number: Related to NIDR-52

Principal Investigator: Dr. D. R. Bergsma

Other Investigators: Dr. L. F. Mills

Cooperating Units: This project is an outgrowth of another NIH project, namely Serial No. NIDR-52 (66); "Production of 'Elfin' Facies and Abnormal Dentition by Vitamin D_2 during Pregnancy: Relationship to the Supravalvular Aortic Stenosis Syndrome."
Principal Investigator: Dr. L. F. Mills, NIDR
Cooperating Unit: Dr. W. F. Friedman, National Heart Institute, Cardiology Branch

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description:

Objectives:

1. To differentiate the rates of spontaneous (primarily genetic) buphthalmos in rabbits and that caused by exposure to vitamin D *in utero* (effects may be cumulative).

2. To establish the dosage levels of vitamin D required to produce this defect.

3. To correlate these findings with those of Drs. Mills and Friedman defining the relationship of vitamin D to elfin facies, abnormal dentition and the supravalvular aortic stenosis syndrome, see above.

Methods Employed:

One normal male and one buphthalmic male were obtained as stud sires. Four normal females and two buphthalmic females were obtained as young breeders. Three major genetic types of animals are being produced: (1) those with both parents normal, (2) those with one parent affected, and (3) those with both parents affected. After a sufficient number of genetically defined animals have been produced in each group to serve as controls, selected females will be given progressively higher doses of vitamin D during pregnancy. Rates of buphthalmos and other abnormalities such as premature closure of fontanelles, malocclusion, and mortality rate will be compared for each group with appropriate controls.

Major Findings:

1. In November, 1967, Dr. Mills observed that three New Zealand white male rabbits, who had been exposed to high doses of vitamin D_2 *in utero*, had unusually large eyes. Further examination revealed that each of these animals (who all had different mothers) had elevated intraocular pressure. Although enlarged eyes with elevated intraocular pressure ("juvenile glaucoma" or "buphthalmos") does occur as an irregular recessive trait among NIH rabbits, the high incidence in those exposed to the vitamin D and its absence in a small control group was considered suggestive enough to warrant a controlled study. That study is this project.

2. To date the project is still in the preliminary stage of producing an adequate number of genetically defined animals. No exposure to vitamin D has been attempted. Therefore there are no new major findings.

Significance to Dental Research:

The relationship between exposure to vitamin D and genetic background in producing the several abnormalities mentioned above is being studied in rabbits as an experimental model of these abnormalities in the human. For example, there is some evidence that increased vitamin D intake during pregnancy may be partially responsible for the increased incidence of the supravalvular aortic stenosis syndrome observed in humans in the past 25 years. Moreover, the causes of buphthalmos in humans are undefined, but there is evidence that both this and other types of glaucoma have a genetic component. Finally, vitamin D has been shown to produce dental malocclusion in rabbits. Therefore, this project combines study of the dental and genetic programs of NIDR.

Proposed Course of Project:

Initially problems of offspring survival delayed the project. The major factors adversely influencing offspring survival have been identified and eliminated. It is anticipated that the project will reach the experimental level described under methods during the coming fiscal year.

Part B not included.

182

Serial No. NIDR-36 (62)
1. Human Genetics
2. Developmental Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Saliva Study

Previous Serial Number: NIDR-93 (c)

Principal Investigator: Dr. R. O. Wolf

Other Investigators: Mr. L. L. Taylor

Cooperating Units: None

Man Years:

Total:	2 1/4
Professional:	1
Other:	1 1/4

Project Description:

Objectives:

1. To use various analytical techniques which have inherently varying resolving powers to determine and define salivary components.

2. To define normal variation of salivary components in normal individuals, as related to environmental conditions at the time of collection.

3. To relate salivary components to genetic control. This includes genetic systems, such as secretor factor, as well as other, unknown but suspected, factors influencing salivary proteins.

4. After methods are perfected and normal bases are established, the salivary components of selected disease entities will be studied.

5. Determine if human salivary isoamylases are under genetic and/or physiological control.

6. Study possible genetic/environmental control of muramidase in saliva and other body fluids.

7. Further investigation of factors in parotid saliva which correlate positively with the rate of calculus formation.

8. Continue development of salivary isoenzyme detection techniques.

Methods Employed:

1. Amyloclastic method of saliva serum and urine isoamylase detection after polyacrylamide gel electrophoretic separation.

2. Muramidase isoenzyme detection in saliva, serum and urine after disc electroporetic separation.

3. Centrifugal ultrafiltration for separation of dental calculus-correlated parotid biuret positive material.

4. Saliva fractionation by column chromatography and gel filtration.

5. Schneyer segregators and Carlson-Crittenden cups are used for the collection of segregated saliva.

Patient Material:

1. Normal control patients of the Clinical Center (NIH permission for the study of Normal Control Patients has been obtained).

2. Selected Clinical Center patients.

3. Individuals from genetically defined populations.

4. Outpatient volunteer families (obtained and administered through the Normal Volunteer Patient Section).

Major Findings:

Human parotid and whole saliva have been shown to be polymorphic and attempts to discover the control of the variation are underway with the major emphasis on the genetic control.

The isoenzyme technique utilized for the demonstration of amylase isozymes has been further developed for the demonstration of lysozyme isoenzymes. A study of the polymorphism of both amylase and lysozyme are in progress for the human body fluids: saliva, serum and urine.

An ultrafilterable biuret positive material was reported found in human parotid saliva which was positively correlated with the rate of dental calculus formation.

Significance to Dental Research:

Two new areas for dental genetic research are being utilized in the study of the control of the polymorphic states of isoamylases and isolysozymes. A hint that a small protein-like molecule in human parotid saliva has something to do with dental calculus formation. Further elucidation of salivary components and their relationships to oral health.

Proposed Course of Project:

1. To detect and define possible gene-controlled salivary components which, in turn, may lead to the definition of new metabolic pathways or corroborate known ones.

2. To investigate batch separation of saliva proteins by continuous flow electrophoresis and gel filtration techniques.

3. Conduct further genetic/environmental studies on human salivary isoamylases and isolysozymes.

4. Corroborate and study the calculus correlated component of human parotid saliva.

Part B

Publications:

Wolf, R. O. and Taylor, L. L.: Isoenzyme demonstration technique. Am. J. Clin. Path., June 1968 (in press).

Serial No. NIDR-37 (58)
1. Human Genetics
2. Population Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Genetic Studies of Oral Clefts and Other Major Congenital Malformations

Previous Serial Number: NIDR-91 (c)

Principal Investigator: Dr. J. D. Niswander

Other Investigators: Mr. C. J. MacLean

Cooperating Units: PHS Division of Indian Health; Lancaster Cleft Palate Clinic, Lancaster, Pennsylvania; Epidemiology Branch, NCI; School of Public Health, University of Hawaii.

Man Years:

Total:	3 1/4
Professional:	3/4
Other:	2 1/2

Project Description:

Objectives: Subproject A

1. To determine if there are physical stigmata associated with cleft palate, familial in nature, that may be useful in:

   a. defining etiologically different defects or syndromes now lumped together.

   b. clarifying the role of genetic (and/or environmental) factors in the genesis of certain clefts. Further, to determine the complexity of the genetic systems involved, e.g., monomeric or polygenic, and to study inheritance of malformations by the use of segregation analysis.

2. To study genetic "fitness" associated with oral clefts through:

   a. reproductive performance of couples who have produced one or more malformed children.

b. study of the survival of children with clefts. An associated objective is to identify the causes of death among those affected individuals who have died and to compare these with the general population. (This aspect of the project has been inactive over the last year due to a one year leave of absence of Dr. Stark.)

3. To determine the frequency of oral clefts in various population groups, as well as genetic and environmental factors important in their etiology.

Methods Employed:

Detailed genetic, social, medical and reproductive history is being obtained on 100 families with oral clefts and 100 controls in Lancaster, Pennsylvania. All family members receive physical examination (primarily limited to head and neck region), tests of visual and auditory acuity and a mid-facial laminograph. Dermatoglyphic patterns and dental casts are also obtained. These data are being analyzed from the standpoint of answering a number of specific questions encompassed under objectives 1a, 1b and 2a.

All children born in Michigan between 1950 and 1960 are being followed to determine the fact of death, age and cause. This aspect involves mainly the use of birth and death certificates and will provide information pertinent to the mortality component of fitness of individuals with clefts.

Data for all cleft births occurring in Montana over a ten year period have been obtained. Analysis will be directed toward the identification of temporal and spatial clustering of cleft births. Also available is data on over 400 families with HL $\pm$ CP from Utah. These data are being used for genetic segregation analysis.

Major Findings:

1. Significantly increased inner canthal distance among affected individuals from families with multiple cases of HL $\pm$ CP. No significant changes in interocular dimensions were found, however, among individuals without clefts in these families.

2. No significant increase in minor abnormalities of the palate and nasal cavity were found in relatives of cleft patients using frontal roentgenographic laminography. This finding is contradictory to other reports in the literature.

3. Dermatoglyphic patterns show greater asymmetry among familial case of HL $\pm$ CP than the control, isolated cleft palate, or sporadic harelip.

4. There is a similar increase in dental asymmetry among the familial cases of harelip ± cleft palate.

5. Analysis of pedigrees shows a significant increase in the frequency of other major congenital anomalies among relatives of children with harelip and/or cleft palate in contrast to the control families. These findings are in contrast to the considerable data which shows a higher frequency of associated anomalies in individuals with isolated cleft palate compared to individuals with harelip.

6. Segregation analysis of harelip ± cleft palate pedigrees suggests that as many as 60% of the cases may be strongly genetic in origin.

Significance to Dental Research:

Better definition of genetic factors in oral facial defects would be useful for counseling and predictive purposes. Also those entities which are non-genetic or have a large environmental component would define a group in which immediate measures aimed at prevention could be instituted.

Better knowledge of associated disease offers hope for better understanding of etiology and should prove of value in medical management of affected children.

Proposed Course of Project:

Complete analysis of data from Michigan regarding causes of mortality in children with oral clefts. Cluster analysis of the Montana data and additional segregation studies of the Utah data.

Objectives: Subproject B

The purpose of this investigation is to ascertain the total (at birth) frequency of congenital malformation among American Indians as well as frequencies of specific major defects. We will then compare these frequencies to comparable data for other Mongoloid and Caucasian populations, as well as certain primitive Indian groups. A further objective is to determine what differences in malformation rates exist between the major linguistic and tribal groups of American Indians. Other factors being assessed include: degree of Indian blood, parental age, parity, season of birth, geography and certain socioeconomic and cultural variables. These variables are to be related not only to "total" malformation rate but also to certain specific anomalies. An additional objective of this project is to serve as a screening procedure to define unique and specific problems of genetic interest for which further detailed studies may be designed. Studies of factors influencing variation

in birth weight are also being conducted as part of this project.

Methods Employed:

The major portion and nucleus of this study involves the use of birth and pediatric records of the various Indian hospitals. Through the cooperation of the Division of Indian Health, copies of these records are available for all Indian births.

A large body of data exists for comparison purposes on congenital malformations in Japanese. These data have been collected by the Department of Human Genetics at the University of Michigan. This group is presently conducting further studies in Japan to supplement these data as well as to collect other material of genetic significance which can be utilized for comparison. More directly applicable are the studies on primitive Indians being undertaken by the Department of Human Genetics.

Field studies among the Papago Indians have continued. One objective is to examine at one year of age all infants on whom we have birth records. This study will provide information on the adequacy of the newborn examination for detection of congenital malformation. In addition, genetic studies (pedigree analysis) will be undertaken for selected malformations encountered among the Papago. Microphthalmia is one such anomaly.

Major Findings:

1. The frequency of cleft lip with or without cleft palate in American Indians is intermediate between Caucasian and other Mongoloid populations, suggesting a relatively simple effect of genetic admixture. Isolated cleft palate frequencies in the Indian closely approximate those of present Japanese. This finding is not easily explained by any simple biological hypothesis.

2. Anencephaly and spina bifida are similar, in that American Indian frequencies for spina bifida are intermediate between Caucasian and Japanese, whereas anencephaly in the Indian remains close to Japanese in frequency.

3. Variation in birth weight among various Indian tribes correlates well with adult weight and stature. This variation can be related to precolumbian food procurement patterns.

4. Analysis of low birth weight in the Indian suggest that this group can be divided into two groups: (1) "Ordinary" low birth weight, i.e. the lower tail of the normal distribution of birth weight and (2) "Deviant" low birth weight, i.e. those babies whose low weight represents the expression of an abnormal

developmental process. Demonstration of this phenomenon is significant to clinical medicine in refining criteria for prematurity and in establishing guidelines for the management of low weight infants. The method used also presents a model which should prove of value in population studies of factors affecting birth weight.

Significance to Dental Research:

1. Comparison of the data with material being collected on Indians will provide measures of racial variation in human populations. Studies of this nature are of importance in defining the relative role of genetic and environmental factors in the etiology of malformation and, hence, ultimately contribute to the knowledge necessary for control.

2. These data will have further bearing on certain theoretical genetic questions of relevance to the etiology of malformations.

3. Specific oral-facial anomalies will be studied in light of the total picture. Their study, not only as isolated entities but in the framework of malformation in general, may lead to clearer understanding of the genetic factors involved.

Proposed Course of Project:

To continue collection of Indian birth data on a semi-permanent basis. Starting from experience and knowledge gained in this project to extend to other more exhaustive studies of dental and physical conditions in American Indians.

To initiate specific studies on diseases and traits of genetic interest as the specific problems become defined.

Part B

Publications:

Adams, M. S. and Niswander, J. D.: Birth weight of American Indian tribes. Human Biology (in press) 1968.

Adams, M. S. and Niswander, J. D.: Developmental "Noise" and a Congenital Malformation. Genetical Research 10: 313-317, 1967.

Adams, M. S., MacLean, C. J. and Niswander, J. D.: Discrimination between Deviant and Ordinary Low Birth Weight: American Indian Infants Growth. Pediatrics (in press) 1968.

Adams, M. S. and Niswander, J. D.: Health of the American Indian - Congenital Defects. Eugenic Quar. (in press) 1968.

Niswander, J. D.: Laminographic X-ray Studies in Families with Cleft Lip and Cleft Palate. Archives of Oral Biol. (in press) 1968.

Niswander, J. D. and Adams, M. S.: Major Malformations in the Relatives of Oral Cleft Patients. Acta Genet. 18: 229-240, 1968.

Niswander, J. D. and Adams, M. S.: Oral Clefts in the American Indian. Pub. Health Rep. 82: 807-812, 1967.

Report of the Laboratory of Histology and Pathology
National Institute of Dental Research
Summary Statement

The past year has resulted in steady progress in most areas. The most noticeable advance has been the revitalization of the Section of Crystal Chemistry through appointment of a new chief. On the other hand, the program on the morphology of mineralized tissue has suffered through the permanent loss of one and the temporary absence of a second key investigator. An improvement is not expected in the immediate future, although every effort will be made to reinforce this vital area.

For the purposes of the present report the activities of the Laboratory of Histology and Pathology are summarized according to several areas of general interest. The projects from which the report have been gathered together are carried out by staff members alone or jointly, and often in collaboration with workers from other laboratories and institutes. The specialized fields represented include electron microscopy and electron diffraction, microradiography, autoradiography, histochemistry, x-ray diffraction, infrared spectrophotometry and experimental pathology.

Cellular and Extracellular Morphology.

This year all the efforts which have involved the use of the electron microscope and microradiographic methods are described under this heading. Most of the projects represent a continuation of previous studies although in several instances the investigations have progressed to a new level. This year's research into the ultrastructure of the central nervous system, for example, has been directed at the main sensory nucleus of the trigeminal nerve. This nucleus is an amportant link in the chain of events through which complex movements such as mastication, deglutition and those involved in speech are effected. Some of the morphological findings are unique relative to other regions of the central nervous system. It is speculated that they represent part of the morphological basis for the neurophysiologic findings of presynaptic inhibition which characterize this region. One important aspect of these studies has been the perfection of a perfusion technique which has made it possible to obtain satisfactory fixation of the trigeminal nuclear complex routinely.

With minor variation the same perfusion technique has been employed in a study of bone resorption and collagen degradation in the periodontal membrane of various animals exposed to extreme stress. In this investigation, however, the resulting fixation has not been entirely satisfactory, although part of the difficulties may be associated with the handling of the specimens subsequent to perfusion. Several possible sources of error have been explored and it is expected that the studies will result in the development of a reproducible technique for preparing such tissues for electron microscopy.

Most previous work on the ultrastructure of periodontal membrane has been restricted, for reasons of poor fixation, to the examination of remnants on extracted teeth. While this type of specimen has limited value, it has been used successfully in this laboratory in previous ultrastructural studies of epithelial rests from normal periodontal membrane. The studies suggested that although the epithelial cells were relatively undifferentiated they possessed the potential for assuming a more active role. Subsequent histochemical and autoradiographic studies showed that this could be achieved by explanting pieces of human periodontal ligament in a suitable medium. Electron microscopic examination of the explant specimens revealed that the epithelial cells were much more highly differentiated judging from the appearance and organization of their cytoplasmic organelles and inclusions. The demonstration that these cells may respond to environmental stimuli throws new light on their possible role in the formation of odontogenic tumors and cysts.

The ultrastructure of the accessory boring organ (ABO) from drilling muricid gastropod molluscs has been investigated in detail. Although of epithelial origin the secretory cells of the ABO, as are osteoclasts, appear to be involved in hard tissue destruction. The electron micrographs showed that the secretory epithelium was penetrated by a complex system of capillaries, muscles and nerves, which was much more extensive than indicated by optical microscopy. The presence of nerve endings on both muscles and epithelial cells suggested the existence of afferent as well as efferent pathways. Comparison of ABO's from actively drilling and inactive snails failed to demonstrate any major cytological differences. However, more detailed information may be forthcoming from cytochemical studies which have been initiated recently.

The studies on microbial morphology which constitute an important program area within the overall objectives of the laboratory have shown steady progress. One project, the determination of the site of action of complement in the complement dependant serum bactericidal reaction has been concerned primarily with the chemical rather than the structural aspects during the past year. This development has followed logically on last year's demonstration that the endotoxic lipopolysaccharide (LPS) was the site of action in at least 2 gram negative microorganisms. Consequently LPS was isolated from one of the 2 and split into a lipid and a carbohydrate fraction, each of which was interacted with complement. While some technical difficulties have been encountered, the results so far suggest that the uptake of complement by LPS is mediated by the lipid fraction. This conclusion if substantiated will lead to a fuller comprehension of serum-cell interactions.

A second project concerned the formation, localization and nature of sulfur inclusions in certain gram negative bacteria. A combination of methods including autoradiography and freeze etching was utilized. The results indicated that the inclusions actually formed outside the cytoplasm while the enzyme(s) responsible for the oxidation of the sulfides resided in the cytoplasmic membrane. This type of study in which membrane function is correlated with morphological entities is important in the overall understanding of cell-environment relationships under investigation in this laboratory.

Other studies carried out by this group, yet not concerned with microbial morphology, have involved localization of at least 2 peptides in the α1 polypeptide of collagen. The α1 polypeptide chain contains 8 peptides which can be separated chem ically and purified. Solutions of α1 chains and of 3 of the 8 peptides were prepared and ATP was added. The α1 chains readily converted into Segmented Long Spacings (SLS) under these conditions. Peptides CB 6 and CB 8 also formed SLS or rather fragments of SLS. Both exhibited characteristic cross banding patterns which were sufficiently precise to allow identification with similar patterns in the α1 SLS. The third peptide tested formed only poorly identifiable SLS fragments in the initial experiments. While a distribution of the 8 peptides has been proposed on the basis of biochemical considerations, the morphological findings will provide more accurate data. Knowing the exact location of the various peptides will contribute considerably to an understanding of various normal and pathologic processes. An example of the latter would be identification of the exact site of action of collagenase, another major concern of the laboratory.

Today radioactive tracers are used extensively for identification of many substances at the ultrastructural level. The parameters for the use of low energy isotopes are well defined while certain discrepancies have been observed in studies involving high energy isotopes. Since bone seeking isotopes possess a high energy, knowledge of the practical resolution and sensitivity characteristics of such a system is necessary for valid data interpretation. In experimental studies with P^{32}, sensitivity values were found to be higher than those predicted theoretically and they deviated more the smaller the angle of incidence. It was concluded that scattering events were responsible for the responsible for the results and that good correspondence could be obtained between experimental and theoretical data by correcting the theoretical distribution curve for the influence of angle of incidence on emulsion sensitivity.

Instead of isotopes, fissionable elements may be used as tracers. The resolution which can be obtained with such a tracer system is considerably better than that provided by radioautographic methods. In addition it is possible to calculate accurately the amount of fissionable material present from the number of tracks. Certain advances were made toward making this approach a useful biological tool. Methods for registration of the tracks were refined and standardized. Numerous tracks were recorded after bombardment of a suspension of uranium containing hydroxy apatite crystals. On the other hand no tracks were observed over uranium containing bone sections, a failure which may be due to the density of the bone tissue. Work is presently under way aimed at circumventing this difficulty.

Histochemistry.

The major concern in this area continues to be the definition of metabolic parameters in oral and other connective tissues. Particular emphasis is placed on enzymes associated with the breakdown of these tissues; namely collagenase and hyaluronidase. The data have remained consistent with the assumption that the collagenase detected is the one concerned with the normal turnover of collagen, and that increased amounts are associated with pathologic conditions. For example, greater than normal amounts of collagenase

were detected in culture fluids of (1) skins from individuals with amyotrophic lateral sclerosis and certain other neuromuscular diseases and (2) synovia from patients with rheumatoid activity. In the latter instance the amount of collagenase detected correlated with the clinical severity of the disease. Several additional sites of collagenase activity in normal tissue were demonstrated in the past year. Thus the enzyme was detected in culture fluids of normal human articular and growth plate cartilage and bone. Likewise a specific collagenase was found in the granule fraction of human neutrophilic leukocytes. This discovery explains how these cells may contribute to the destruction of collagen in many pathologic states. Further characterization of the enzyme indicated that it is active at pH 7-9 and that it is completely inhibited by EDTA and partially by cystein. Electron microscopic studies coupled with data from acrylamide gels showed that the human collagenase cleaved the collagen molecule into a 3/4 and 1/4 piece, an action which is similar to that of tadpole derived collagenase, but distinct from that of the bacterial enzyme.

The studies on hyaluronidase involved detection of the enzyme in alveolar macrophages in rabbits. Here as in human gingivae the enzyme manifested a pH optimum different from that of bacterial hyaluronidases. The detection of hyaluronidase in various tissues explains how hyaluronic acid, chondroitin sulfate A and chondroitin sulfate C may be degraded in normal and pathologic tissues.

Histochemical studies of enzymes with activities less directly identifiable with specific cellular functions were also continued. These enzymes which are concerned with the production of energy, and fatty acid, carbohydrate and mucopolysaccharide metabolism include the phosphates, the est numerous dehydrogenases, and the glycosidases. Both esterase and selected glycosidase activity appeared to be correlated with cellular activity associated with active remodelling of bone and cementum. Most of the esterase activity in rat jaws was demonstrated to be non-specific in nature, the bulk being a B type esterase while only a small amount of C type esterase was identified.

Crystal Chemistry

The activities of the Crystal Chemistry Section can be conveniently categorized into three areas of interest. Two of these areas represent new directions of study for the section.

New research has been initiated in the areas of diffraction of x-rays by biofibers. The broad objective in undertaking these studies is to collect diffraction data from biological fibers under a wide variety of experimental situations and attempt to interpret the x-ray findings in terms of those structural parameters which may be of particular relevance to understanding their biologic role. During the past year this objective has been pursued through two specific lines of investigation. First, a comparative study of the x-ray diffraction properties of hard and soft tissue collagens was undertaken. From data accumulated in this study to date, advances in understanding the role covalent crosslinks may play in defining collagen structure have

been made. Of particular significance was the finding that the ability of chemically denatured collagen to renature was dependent upon the presence of intermolecular covalent cross-links. Bone collagen, which is crosslinked was found to completely renature under conditions where tendon collagen, devoid of cross-links, showed no signs of renaturing.

A second specific line of investigation undertaken was a diffraction study aimed at delineating the structure of the protein components of amyloid tissue. Previous studies in this laboratory and elsewhere have demonstrated that amyloid deposits contain 2 morphologically different structures which do not seem to be interconvertible. One of these appears as a periodic rod, the other and major component is a non-periodic fibril. The discovery that this latter amyloid protein exhibited an x-ray pattern of the cross-β type may have particular importance to dental research. The only other cross-β protein reportedly found in human tissue comprises the major component of the matrix of enamel. The periodic rods proved to have an "amorphous" wide-angle x-ray diffraction pattern. Their structure as observed in the electron microscope suggests, however, that this material may have a small-angle diffraction pattern, a possibility which will be further investigated.

The Crystal Chemistry Section has maintained a long standing interest in biological and synthetic calcium phosphates. This interest, however, has taken a new direction with attention shifting away from crystalline salts and, instead, focusing on amorphous phases. Previous reported studies have demonstrated that the mineral investing hard tissue contains, in addition to crystalline hydroxyapatite, an amorphous calcium phosphate as a major phase. Much of what is known, however, about this amorphous salt has been deduced by inference from its synthetic analogue. Unfortunately, the diffuseness of the diffraction pattern has placed definite limitations on the ability of x-ray analysis to demonstrate whether this synthetic amorphous calcium phosphate is a unique phase or is a cryptocrystalline form of one of the known crystalline calcium phosphates. A new experimental program is being undertaken with the express purpose of more clearly defining the synthetic amorphous salt by employing thermochemical techniques.

Another area of continuing interest to the section has been infrared absorption spectrophotometry of hydroxyapatite and related compounds. During the past year, studies on isotopically substituted apatites have led to some major reassignments of absorption bands in the IR spectra of hydroxyapatite. Another important advancement in the study of the IR spectra of apatite has been the development of a hydrothermal procedure for isotopically labeling PO_4 groups in hydroxyapatite with O^{18}. This procedure should not only prove invaluable in the future study of the IR spectra of apatites but also in expanding the capability of IR spectroscopy for the study of reaction mechanisms and thermal diffusion in apatites. A study was also initiated on characterizing the IR spectra of strontium and various apatites to aid in the assignment of some low frequency bands in the hydroxyapatite spectrum. The object of all these studies is not just to understand the IR spectrum of hydroxyapatite for its own intrinsic interest but to develop this understanding to the point where IR spectroscopy can be of invaluable assistance in the important problem of obtaining information on the effects impurities have on the chemical and physical properties of biological apatites.

Experimental Pathology

This year's studies as were those of the preceding years have been aimed at gaining greater insights into the components of the dento-bacterial plaques, their pathogenic potential, and methods for controlling the deposits and their toxic by-products. Special emphasis has been placed on understanding the mechanism of adhesion between the microorganisms and the surfaces of the teeth. It appears that this, in large measure, is effected through the production of extracellular polysaccharides. Streptococci that produce abundant amounts of dextran and high concentrations of intraplaque acids are conducive to rampant dental decay. When dextranase was added to the diet and/or water of hamsters affected with dental plaque infections produced by dextranogenic streptococci, this type of deposit did not collect on smooth surfaces. As a result dental caries did not occur in these highly vulnerable areas. However, this enzyme did not affect plaque deposits which resulted from the interaction of levanogenic diphteroids and various starches and sugars. Nevertheless preventive treatment aimed at the dispersion by chemical means of adherent plaques on the surfaces of teeth represents a new and possibly major advance in the fight to achieve plaque control.

The importance of appropriate dieto-bacterial challenges in the induction of plaque infections was demonstrated again in studies of experimental caries and periodontal syndromes. After many unsuccessful attempts, cervico-radicular plaque formation and associated periodontal disturbances were induced in hamsters by feeding them a diet containing starch and by inoculating their mouths with human diphteroids. An infectious component was also found to be involved in experimental periodontal pathosis in rats. However, attempts to colonize an established hamster strain of plaque forming microorganism in dogs failed even under favorable dietary conditions. In contrast a high degree of caries activity was induced in rats once considered to be resistant to cavitation by an appropriate dieto-bacterial challenge.

While it may become possible to control smooth surface caries through the use of plaque dispersing agents, prevention of fissure caries may depend on other approaches. One that could be of great value in preventing this type of lesion is the use of sealing agents to seal vulnerable retention sites. Initial experiments with materials containing n-methyl cyanoacrylate were only partly successful because the adhesive did not possess adequate bio-stability. In vitro experiments indicated, however, the effectiveness of this approach. At present other sealing materials are being tested in vitro and in animals before undergoing clinical trials.

Serial No. NIDR-38 (63)
1. Histology and Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Experimentally Induced Enamel Defects

Previous Serial Number: NIDR-15

Principal Investigator: Dr. M. U. Nylen

Other Investigators: None

Cooperating Units: Dr. K.-A. Omnell, Royal Dental School, Malmo, Sweden

Man Years:

Total:	1
Professional:	1/4
Other:	3/4

Project Description:

Objectives:

Previous experiments have shown that intraperitoneal injections of tetracycline hydrochloride (TC) in rats result in both hypoplastic and hypomineralized enamel defects. The purpose of the continued studies was:

1. to compare the effect of oxytetracycline (OTC) with that of TC.

In the course of these experiments the enamel was found to be labeled by the antibiotic in an unusual yet consistent manner. Because of these observations new experiments were designed, the purpose of which was:

2. to investigate in more detail the relative staining behavior of normal and pathologic enamel.

Methods Employed:

1. Twenty Sprague-Dawley rats, approximately 75 days old, were used as experimental animals. Half of the rats were given 25 mg/kg body

weight of TC on day 0, an equivalent dose mol per mol of OTC on day 5, followed by 150 mg/kg body weight of TC on day 10 and an equivalent dose of OTC on day 15. In the remaining animals the sequence of administration was reversed with a low dose OTC starting the injection series. All the rats were sacrificed 10 days after the last injection and 50 micron midsagittal ground sections were prepared from the upper incisors and examined under ultraviolet light and microradiographically.

2. Sprague-Dawley rats, approximately 75 days old, were injected subcutaneously with NaF in doses ranging between 80 and 125 mg/kg body weight. Intraperitoneal injections of 125 mg/kg body weight TC were administered 5 and 10 days later. The animals were sacrificed 1, 5 or 10 days after the TC injection and upper incisors were prepared for examination under UV light and for microradiography as described under 1.

Major Findings:

Neither TC nor OTC produced enamel defects consistently at the lower dose levels. On the other hand both were capable of producing enamel defects at the higher dose levels. On the basis of differences in the severity of the response the lesions were divided into 3 groups. Group 1 comprised the least severe lesions, i.e., those in which the incremental band constituted the only "structural" disturbance. Group 3, on the other hand, exhibited lesions including gross hypoplastic defects, in addition to the incremental line. A comparison of the effect of the high doses of TC and OTC on the enamel showed that while 15 of the total 20 animals developed group 3 malformations following the TC administration, only 1 such lesion was associated with the OTC injections. In contrast only 1 TC, but 17 OTC lesions, were classed in group 1.

Under U.V. light, 4 fluorescent lines, each representing one injection were present in the dentin. The color of the fluorescence varied; the OTC bands yielding a greenish yellow, and the TC bands a golden yellow fluorescence. In the enamel, the incremental bands were generally non-fluorescent. Of the gross hypoplastic lesions, only those associated with the first high dose injection were labeled, while those caused by the second administration were non-fluorescent. Although all of the labeled lesions were the result of a TC injection, the color of the fluorescence seemed greenish rather than golden yellow.

These findings suggested that preexisting lesions were labeled permanently by subsequent administrations of tetracycline while the initial label was lost as it is in normal developing enamel. Since the hypoplastic lesions are markedly hypomineralized and contain large intercrystalline spaces, as shown by electron microscopy, they are readily accessible to the antibiotic at a level of development when the surrounding normal enamel is too highly mineralized. Why the label should be retained permanently at this stage of development and not initially remains to be explained, although it may be related to crystal growth phenomena.

The ability of the fluorphor to locate permanently in areas of defective enamel was borne out by the 2nd series of experiments. In these studies it was found that gross hypoplastic lesions, created by subcutaneous injections with high concentrations of NaF, were the only portions of the enamel labeled permanently by a subsequent administration of tetracycline.

Significance to Dental Research:

The tetracyclines are some of the most commonly used antibiotics. Previous investigators have shown that although all of the commercially available products may discolor the developing teeth permanently, the effect of oxytetracycline is somewhat less severe. The present findings that oxytetracycline also is less apt to produce hypoplastic enamel lesions give added significance to the previous observations.

The findings that defective enamel may be labeled during later administration of tetracycline while tetracycline induced defects lose their initial label are of importance in determining the origin of enamel lesions. Only those associated "geographically" with fluorescent dentin lines may be attributed to the antibiotic. These findings further indicate that incorporation of tetracycline in the enamel is not limited to the formative stage, but may occur during the entire pre-eruptive phase provided defective zones are present.

Proposed Course of Project:

The study has been completed and is in the process of being written up.

Part B

Publications:

1. Lofgren, C.-J., Omnell, K.-Å., and Nylen, M. U.: Effect of Intraperitoneal Injections of Tetracycline Hydrochloride and Oxytetracycline on Forming Enamel of Rat Incisors. Accepted for publication in Calcified Tissue Research.

2. Nylen, M. U.: Recent electron microscopic and allied investigations into the normal structure of human enamel. Int. Dent. J. 17:719-733, 1967.

Serial No. NIDR-39 (59)
1. Histology and Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Collaborative Projects and Training Activities

Previous Serial Number: NIDR-16

Principal Investigator: Dr. M. U. Nylen

Other Investigators: see below

Cooperating Units: see below

Man Years:

Total:	2 1/4
Professional:	1/2
Other:	1 3/4

Project Description:

Objectives, Methods Employed, and Major Findings:

Over the years a number of collaborative projects involving the use of biophysical methods have been undertaken upon the request of investigators in other laboratories. Many of the problems have been directly related to the general program of this laboratory and have constituted a sizeable portion of our activity. The principal projects are listed below.

Collaborative Studies:

1. Studies of calcareous corpuscles in tapeworms. X-ray diffraction of corpuscles from different tapeworm species after various degrees of heating. In addition the uptake of phosphate by isolated corpuscles is investigated using x-ray diffraction methods. Uninterrupted heating of calcareous corpuscles of Taenia tacniaformis for 180 days at 150 C resulted in a very faint pattern possibly indicating the formation of dolomite. A much more distinct pattern was visible when the temperature was increased to 180 C, the heating period lasting from 4 to 5 days. At 300 C dolomite was formed after 15 minutes and at 450 C after 5

minutes although some decomposition took place at the latter temperature as indicated by the slow appearance of calcium carbonate and magnesium oxide patterns. Relatively large amounts of phosphate were incorporated in calcareous corpuscles incubated in vitro in a phosphate solution. The diffraction studies indicated that the phosphate gave rise to apatite formation. With Dr. T. von Brand, Laboratory of Tropical Disease, NIAID. To be continued.

2. Electron microscopic studies of the accessory boring organ of molluscs. This past year the work has been concerned largely with studying the relationship of the epithelial cells to each other and to the muscles, blood vessels and nerves, which permeate the tissues. The epithelial cells are arranged in groups, each of which is surrounded by a basement membrane. The cells are very irregular especially in the basal half where they interdigitate profusely with each other. Numerous pericyte-lined vessels are located between the epithelial cell groups. They are most prominent basally but can be followed almost to the distal microvillar zone, where they appear to be open to the interstitial spaces. As a consequence hemocyanin is found not only inside the vessels but also in all the spaces between the vessels and the epithelial cells. A much greater number of hemocyanin molecules is seen in the active than in the inactive organs, probably reflecting the greater demand for oxygen in the former instance. Muscles too are found between the epithelial groups where they frequently assume an orientation perpendicular to the long axis of the secretory cells. Groups of nerves seem to follow the muscles into the tissue while others appear to be located on the epithelial side of the basement membrane surrounding the epithelial cell groups. Axons of the latter can be traced distally to the immediate vicinity of the microvillar border.

Apart from mapping this complex tissue, additional information has been obtained concerning the cytoplasmic organization of the epithelial cells. Of particular interest has been the finding of numerous large bodies which appear to be autophagic in nature. In order to identify these bodies better, cytochemical studies have been initiated, and the continuation of the project will center around this aspect. With Dr. M. R. Carriker, Marine Biological Laboratories, Woods Hole, Massachusetts, and Dr. V. Provenza, Maryland University Dental School, Baltimore, Maryland.

3. Electron microscopic studies of explanted epithelial rests from normal human periodontium. Small root fragments with adherent periodontal membrane were explanted in a medium described by McCoy et al and were harvested at various incubation intervals ranging from 5-12 days after explantation. Experimental and control specimens were fixed in glutaraldehyde followed by osmic acid and embedded in Maraglas. Examination of lead stained thin sections revealed many distinct differences between the cultured and the control material.

The cytoplasm is more abundant in the in vitro cells and contains an increased quantity of better defined organelles. Thus the mitochondria are larger with more distinct outer membranes and internal cristae. The Golgi complex which is missing in the control is very prominent in the experimental material. The endoplasmic reticulum is also enhanced. The profiles, however, are only partially covered by ribosomes suggesting a transitional stage between smooth and rough surfaced endoplasmic reticulum. Free ribosomes as well as polyribosomal aggregates are also more numerous in the in-vitro cells. Of the inclusions lysosomes and lipid bodies are more frequent in the explants than in the resting cells while the opposite is true of glycogen and tonofilaments. Additional evidence of an increased metabolic activity is presented by the change in cell contour. In the cultured cells the plasma membranes feature numerous microvillar projections which interdigitate with those of the adjacent cells while those in the control are much more regular.

Since both histochemical and autoradiographic studies have indicated that the epithelial rests assume a more active state following explantation, the morphological changes are not unexpected. The findings serve to emphasize, however, once more the relationship between cell activity and cytological differentiation. The fact that these cells have the capacity to become more active is of interest to considerations of their possible contribution to the formation of cyst linings and even to odontogenic tumors. The project which has been carried out in collaboration with Dr. H. A. Zander and Dr. H. E. Grupe, Eastman Dental Center, Rochester, New York, is completed and the results are in the process of being written up.

4. A microradiographic study of the zone of calcification which in man attaches the articular cartilage to bone. Variations in this interface with age, species and disease as well as in experimentally induced remodeling are being investigated. Preliminary results have indicated that in man the calcified layer is more highly mineralized than the subchondral bone. With Dr. E. D. Eanes, LHP, NIDR and Drs. L. Sokoloff and W. Green, LEP, NIAMD. To be continued.

Training Activities:

The following persons have received training in electron microscopy and associated techniques.

Dr. Lenore Disher, School of Dentistry, University of California, San Francisco Medical Center and a U.S.P.H.S. post-doctoral fellow has been a guest worker in the laboratory since February 1, 1968.

Dr. A. Carl Verrusio, Section of Pharmacology, Laboratory of Biochemistry, N.I.D.R., who has been with the laboratory as a trainee since January 1968.

Significance to Dental Research:

The importance of the collaborative and training efforts is self-evident. Through the collaborative projects, the laboratory staff gains a broader experience in the general field of biophysical instrumentation, which together with the accumulated data, frequently serve as a basis for new experimental approaches to problems more directly related to the oral tissues.

Proposed Course of Project:

As indicated above, collaborative work will continue on numerous problems. Training activities will also be continued.

Part B:

Publications:

1. von Brand, T., Nylen, M. U., Martin, G. N., and Churchwell, F. K.: Composition and crystallization patterns of calcareous corpuscles of cestodes grown in different classes of hosts. J. Parasitol. 53:683-687, August 1967.

Serial No. NIDR-40 (67)
1. Histology and Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Studies on Bone Resorption and Collagen Degradation due to Pressure

Previous Serial Number: None

Principal Investigator: Dr. Jens Waerhaug

Other Investigators: Dr. M. U. Nylen

Cooperating Units: Dr. W. Titus, Laboratory Aids Branch, Division of Research Services

Man Years:

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives:

To study the changes which occur in the periodontal membrane of teeth exposed to extreme stress.

Destruction of the bone that supports the teeth is one of the most spectacular features of periodontal disease. However, until about a decade ago the mechanism by which bone was resorbed was highly speculative. The application of electron microscopy to the study of osteoclastic resorption has contributed substantially to our general understanding of this process, but it still remains to be shown to what extent the same series of reactions take place in the periodontal membrand under varying degrees of stress. The purpose of the present experiments is to create conditions in the periodontal membrane of experimental animals that correspond to the concept of extreme trauma from occlusion in man.

Methods Employed:

Dogs, cats, and rats were used as experimental animals. In the larger animals trauma to the periodontal membrane was created by cementing a high crown onto the lower first molar on one side, allowing the antagonists to occlude with a shelf on the crown. In the rats all the

upper molars on one side and one or two molars on the other side were extracted on the assumption that the remaining molars which still had antagonists would be subjected to excessive stress.

The experimental period varied from 2 weeks in the dogs and cats to 5 days in the rats. On the day of sacrifice the animals were anaesthetized, their thoracic cavity opened, and a canula was inserted through the left ventricle into the ascending aorta. In the larger animals the subclavian arteries and the descending aorta were ligated immediately prior to insertion of the canula. In the rats the descending aorta was clamped with a hemostat. In all animals the right atrium was opened as soon as the canula was inserted to allow free drainage of the fluid.

The perfusate consisted in most instances of phosphate buffered glutaraldehyde in amounts which depended on the size of the animal. One animal, however, was perfused with phosphate buffered osmic acid only and another one was perfused first with glutaraldehyde and subsequently with osmic acid.

Immediately after perfusion the stressed teeth were excised and cut into smaller pieces either using a rotating saw blade or a water cooled, rotating rubber banded carbide wheel. In all instances final fixation was effected by immersion of the small pieces in phosphate buffered osmic acid over night.

Dehydration and embedding in epon followed standard procedures for hard tissues. Thick sections were cut with a diamond knife, stained with toluidine blue and examined under the optical microscope. Thin sections were likewise cut with a diamond knife from selected areas, picked up on carbon covered substrates, stained with lead citrate and examined in a Siemens Elmiskop 1A 6B electron microscope.

Major Findings:

Thick sections revealed the presence of numerous osteoclasts and disappearance of collagen structure. Unfortunately the results at the ultrastructural level have been disappointing in that most of the samples failed to exhibit satisfactory fine structural details. It seems obvious that perfusion alone rarely is sufficient to achieve a satisfactory degree of fixation. To what extent the damage is a result of the treatment which followed perfusion and/or failure of the fixative to reach the pertinent areas during the subsequent immersion phase are being investigated. Since it has been shown that glutaraldehyde fixed tissues remain osmotically active, the cutting-up of the specimens may well be the critical phase.

Significance to Dental Research:

A further clarification of the mode of action of bone resorbing cells has considerable didactic importance. Bone resorption has been and still is, a poorly understood biological process. In addition very little is known about the fine structural changes which occur in the periodontal membrane under pathologic conditions. The development of useful preparatory techniques which will allow such studies to be carried out in a reproducible and meaningful manner is a prerequisite for any advances in this area.

Proposed Course of Project:

The investigator's tenure of appointment ceases in August 1968. The experimental work will be continued to that date, while examination of the material and analysis of the results will go on past that date.

Part B: not included

Serial No. NIDR-41 (64)
1. Histology and Pathology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: X-ray Diffraction Studies on the Effect of Fluoride on Bones, Teeth and Synthetic Compounds

Previous Serial Number: NIDR-19

Principal Investigator: Dr. Paul D. Frazier

Other Investigators: None

Cooperating Units: Dr. J. E. Seegmiller, NIAMD, NIH; Dr. P. Carbone, NCI, NIH; Dr. H. Schraer, Penn State University, University Park, Penna.

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

These studies are being conducted to determine more completely the effect of fluoride on crystal chemistry of bones and teeth. The influence of fluoride on nucleation, size, shape, strain and lattice constants of apatite crystal systems are also being investigated.

Methods Employed:

Wide-angle X-ray diffraction techniques are employed using film and electronic detectors. Electron microscopy and contact microradiography were also used.

Major Findings:

The wide variations in crystallite size of enamel reported in the literature suggested that the breadth of the diffraction profiles used to make the calculations was affected by preparation techniques. A study was therefore conducted to determine the influence of preparation methods on profile breadth. Broadening was observed in enamel

ground with high and low speed dental instruments when compared to counter-part enamel ground under controlled conditions in a ball mill. Prolonged ball grinding also caused severe line broadening. A single crystal of mineral hydroxyapatite was not damaged when it was prepared by grinding with low speed dental instruments. The ease with which enamel is damaged may be related to its composite structure as opposed to the mineral apatite. Electron microscopy studies of the damaged enamel indicated that 1) there is a significant number of broken crystallites, 2) the number increases as does line broadening with increased grinding and 3) the size of unbroken clumps of crystals was far smaller than individual particles of mineral hydroxyapatite prepared under identical conditions.

X-ray diffraction studies of human enamel containing varying amounts of fluoride indicate that there is a measurable decrease in line breadth of the 002, 211, and 300 sets of planes as fluoride content increases. The correlation coefficients for 002, 211, and 300 were -0.9, -0.7 and -0.7 respectively. The highest degree of association between fluoride and change in line breadth was for the 002 peak; earlier investigations in this laboratory have indicated no such relationship for bone.

Electron probe analysis of incipient carious lesions has shown that the probe is a useful instrument for detecting Ca to P ratios on a microscopic level. Demineralized areas in the lesion have been observed with higher than normal Ca/P ratios; there are also hypermineralized areas with lower than normal Ca/P ratios.

Three methods of measuring changes in bone crystallinity have been developed which will be more universally applicable than the template method originally developed in this laboratory. The primary advantages of these techniques are all based on direct measurements or data taken directly from the instrument. This eliminates assumptions needed for constructing the original template and the data can be fed directly into a computer if desired.

Significance to Dental Research:

Enamel crystallite size values obtained from severely damaged enamel were 1/4 that of the a-axis dimension and up to 1/6 that obtained for the c-axis of non-damaged counter-part enamel.

These findings support the argument that the wide variation in crystallite size values in the literature could be related to variations in sample preparation. Many investigations are using these same grinding and collecting techniques for other physical studies. However, investigations have not yet been made which might associate the "damaged" fraction of enamel observed by X-ray diffraction and electron microscopy with variation in 1) chemical composition, 2) density, 3) and/or other physical properties.

The finding that increased fluoride incorporated into bone mineral improves its crystallinity has been extended to human enamel. The complete significance of these findings cannot be determined until experimental evidence is obtained separating the influence of strain and size on line broadening.

The results of the electron probe study of carious lesions indicate that future studies in conjunction with electron microscopy will give information concerning the chemical as well as morphological changes within the lesion.

The new methods developed for studying bone will facilitate rapid accurate studies of the age effect on crystallinity in high and low fluoride areas. In addition the technique can be applied to animal studies where age and fluoride feeding are controlled.

Proposed Course of Project:

Principal investigator has been attending Graduate School at the University of Washington, Seattle, Washington, since September 1967. The project is discontinued for the duration of training period except for writing up some of the data.

Part B:

Publications:

1. Frazier, P. D. Adult human enamel. III An electron microscopic study of crystallite size and morphology. Accepted for publication in Ultrastructure Research.

2. Frazier, P. D. and Wong, V. G. Cystinosis: Histologic and crystallographic examination of crystals in eye tissues. Accepted for publication in Archives of Opthalmology.

211

Serial No. NIDR-42 (66)
1. Histology and Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Fine Structural Studies of the Main Sensory Nucleus of the Trigeminal Nerve

Previous Serial Number: NIDR-21

Principal Investigator: Dr. Stephen Gobel

Other Investigators: Dr. Ronald Dubner, Section of Physiology, NIDR

Cooperating Units: None

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives:

1. To determine the normal morphological characteristics of the neurons and glial cells comprising the main sensory nucleus.

2. To determine the normal synaptic relationships between the neurons of the main sensory nucleus and the axons entering this nucleus.

Methods Employed:

A procedure for fixation of the brain stem of adult cats by vascular perfusion has been developed. Before opening the thoracic cavity ligatures are placed around the subclavian arteries distal to the emergence of the vertebral arteries and around both common carotid arteries. Just prior to perfusion the above arteries are tied off. A canula is then inserted through the left ventricle into the ascending aorta and tied in place. The right atrium is opened to allow for outflow of the perfusate and the descending aorta distal to the emergence of the left subclavian artery is clamped off. The perfusion of the brain stem takes place through the vertebral arteries. By restricting the flow of perfusate to the vertebral arteries it has been possible to

obtain consistently preparations of the trigeminal nuclear complex suitable for fine structural studies. The perfusate consists of phosphate buffered glutaraldehyde followed by dichromate buffered OsO_4 or dichromate buffered OsO_4 alone. After fixation the pons is divided into right and left halves. Each half is cut anteroposteriorly into 1 mm blocks and flat embedded in an epoxy resin. One micron sections are cut from these blocks in order to locate the main sensory nucleus. The nucleus is then divided into 4 quadrants, reembedded and subsequently prepared for electron microscopical study.

In an auxillary technique formalin or glutaraldehyde fixed material is impregnated with $AgNO_3$ by the rapid Golgi method. Thick sections (100u) are cut on a freezing microtome. Material treated in this way is used to provide information on the extent of branching and dispersion of the dendrites in the main sensory nucleus.

Major Findings:

The neurons of the main sensory nucleus are the second neuron in a chain of three leading from the orofacial area to the cerebral cortex. Their cell bodies range from 10-30 u id diameter and are apposed by dendrites, myelinated and unmyelinated axons, astrocytic processes and cell bodies of neurons, astrocytes and oligodendrocytes. The density of axonal boutons synapsing on the surface of these cell bodies is relatively low. A few spines protrude from the cell bodies and usually have synapses on them.

The neurons of the main sensory nucleus contain 3 or more main dendrites. After a short distance they branch secondarily. Some of these secondary branches extend more than half the width of the nucleus. On some proximal branches the density of axonal boutons can be quite dense; four or five adjacent boutons may synapse on them. Occasional short spines occur along the dendrites and protrude into axons with which they synapse.

Several distinct morphological types of axons have been identified. One type, having large synaptic segments which contain large diameter synaptic vesicles, forms numerous axodendritic and axo-axonic synapses. The synaptic segments of this axon may have 2 or more axo-axonic synapses on them as well as sending out projections which form synapses by invaginating into other axonal types. Two types of axons which consist of smaller synaptic segments and contain smaller diameter synaptic vesicles enter into axo-axonic synapses with each other and with large diameter axons previously described. Axo-axonic synapsis is often made by interdigitation of small spine-like projections. The involvement of three axons in axo-axonic synapses by means of projecting into one another has not been observed previously in the central nervous system. This series of axo-axonic synapses probably represents a part of the morphologic basis for the neurophysiologic findings of presynaptic inhibition which characterize this region of the central nervous system.

Significance to Dental Research

The trigeminal nerve conveys to the central nervous system information pertaining to tactile discrimination, pain and temperature sensation from the teeth, periodontal ligament, oral cavity and anterior two thirds of the face as well as proprioceptive information from the periodontal ligament and muscles of mastication. In the main sensory nucleus as well as other parts of the trigeminal nuclear complex such information is passed on to a set of neurons which serve as relays to the cerebral cortex via the thalamus. However, before this information leaves the trigeminal nuclear complex it is modified and integrated with information coming from diverse parts of the central nervous system, i.e., cerebral cortex, spinal cord, reticular formation and different parts of the trigeminal system. It is through such integration of information that complex movements such as mastication, deglutition, turning of the head and those involved in speech can be effected. An appreciation of the fine structural basis for this integration is essential for comprehending orofacial function.

Proposed Course of Project:

A. Separate lesions will be made in the trigeminal nerve, spinal nucleus of V and the cerebral cortex. The interrupted axons will be allowed to degenerate. By identifying electron microscopically the degenerated axonal boutons it will be possible to conclusively demonstrate the manner in which axons from the above sources synapse with cells of the main sensory nucleus and whether they form axo-axonic synapses with each other. It is planned to establish whether there is synaptic interaction between axons from the above-mentioned sources.

B. Long range plans involve extending fine structural studies to the spinal nucleus of the V, motor nucleus of the V, and the mesencephalic nucleus as well as the mode of origin of the trigeminal nerve in relation to odontoblasts.

Part B:

Publications:

1. Gobel, S.: Electron microscopical studies of the cerebrellar molecular layer. J. Ultrastructure Research, 21:430, 1968.

214

Serial No. NIDR-43 (62)
1. Histology and Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Bacterial, Viral and Macromolecular Structure-function Relationships

Previous Serial Number: NIDR-22

Principal Investigator: Dr. H. A. Bladen, Jr.

Other Investigators: Dr. G. Hageage, Dr. K. A. Piez

Cooperating Units: None

Man Years:

Total:	2 3/4
Professional:	1
Other:	1 3/4

Project Description:

Objectives:

As in the past, studies have been primarily an attempt to determine possible relationships between form and function of biological materials. Recent interests in complex biological problems have dictated the necessity for utilizing techniques in disciplines other than electron microscopy. These include biochemistry, immunology and microbiology. The problems presently under investigation in this laboratory are:

1. To determine the chemical as well as morphological site of action of serum complement on the bacteria cell and its endotoxic LPS.

2. To determine the morphological relationship of specific collagen peptides to the collagen molecule itself.

Methods Employed:

1. Endotoxic lipopolysaccharide (LPS) of V. alcalescens was isolated by phenol-water extraction of whole cells. Final lyophilization of the water phase resulted in LPS which had a characteristic structure when negatively stained.

The lipoidal moiety of the LPS, designated as Lipid A, was prepared by refluxing LPS with 0.1 N HCl for 1 hour. The milky reaction mixture was then extracted several times with chloroform, washed with water, and finally extracted with hot acetone. This was dried to give a brown, waxy material which was lipid A.

Utilization of serum complement by V. alcalescens LPS was demonstrated by initially reacting known amounts of LPS with complement, then determining residual complement by the usual hemolytic assay procedure.

Complement utilization by Lipid A was difficult to quantitate due to the insolubility of lipid in non-organic solvents. The best results were obtained by sonication of the lipid fraction followed by filtration through an 8 u milipore filter. The relatively uniform suspension of Lipid A which resulted was then reacted with a known amount of serum complement and, after an incubation period, residual complement was determined by hemolytic assay.

2. Purified collagen peptides, the amino acid composition of which was known were supplied by the Laboratory of Biochemistry, NIDR. Segmented Long Spacings (SLS) were prepared from these specimens by dialyzing against 0.05M acetic acid and then reacting with ATP. The reaction mixtures became turbid after intervals of time ranging from 3 to 30 minutes. Occasionally SLS were formed by dialyzing the specimens in acetic acid against ATP. After formation of SLS, preparations were positively or negatively stained with phosphotungstic acid and examined in an AEI 6B electron microscope.

Major Findings:

1. Previous studies in this laboratory have demonstrated that guina pig serum produces lesions approximately 90 A in diameter on the cell surface of E. coli and V. alcalescens. These lesions are identical in morphology to lesions present on erythrocyte membranes after action of complement.

Similarly, the endotoxic LPS of these organisms reveal lesions after reaction with serum complement. Continued experimentation demonstrated these lesions to be dependent on the presence of serum complement.

In the present phase of the study, LPS was fractionated into the lipoidal and carbohydrate fractions. Mild hydrolysis of LPS yielded a carbohydrate moiety as well as a lipid, termed lipid A. Initial studies which were concerned with the carbohydrate moiety demonstrated that 30-40% of the available complement was taken up by the carbohydrate. However, chemical analysis showed that the carbohydrate obtained still had considerable lipid present. Strenuous hydrolysis of LPS eventually resulted in a carbohydrate fraction in which lipid could not be detected

by methods used. This carbohydrate showed negligible complement uptake. It should be noted, however, that such harsh treatment of the carbohydrate most certainly hydrolyzed it, so that it bore little resemblance to the carbohydrate moiety of the parent LPS.

Initial experiments with the Lipid A fraction were rather erratic since the Lipid was not soluble in the buffer system used. Sonication resulted in a particulate suspension which was difficult to test quantitatively for complement uptake Some lipid suspensions resulted in practically total uptake of complement while others gave only about 20-30% uptake. For instance, when relative volumes of Lipid A were increased, no increase over 20% utilization was observed, suggesting that the amount of lipid present was very small. Centrifugation of sonicated lipid suspensions also yielded preparations which did not utilize complement quantitatively. This was probably due to the dispersal of existing clumps of lipid. However, sonication followed by filtration through an 8 u milipore filter resulted in rather reasonably straight line relationships. In one experiment, it was determined that 60 ug Lipid A utilized approximately 50% of the available complement. This figure is quantitatively similar to the utilization by 100 ug LPS, 60% of which is lipid.

In another experiment, 100 ug Lipid A was placed on a TLC plate, separated into several spots and eluted as a whole, before being tested for uptake of complement. Approximately 40-45% uptake was determined. These results suggest that all of the uptake of complement by LPS may be due to the lipid fraction.

2. At the present phase of this study, three different peptides, isolated from the $\alpha 1$ polypeptide chain of collagen, have been converted into Segmented Long Spacings (SLS) by the addition of ATP. The structural integrity, i.e. the arrangement of bands at various intervals, along peptides CB6 and CB8 is sufficiently precise as to allow localization of these peptides in SLS prepared from the intact $\alpha 1$ polypeptide.

Peptide CB8 is composed of at least 9 resolvable bands which are identical in width, intensity and separation to 9 bands present in the parent $\alpha 1$ polypeptide SLS. The CB8 peptide begins approximately 380A from the A end of the parent $\alpha 1$ SLS and, with a total length of 870A, comprises 27% of it.

Peptide CB6 has at least six identifiable bands which correspond to six bands with a location near the B end. It is approximately 700 A in length and occupies 22% of the total length of the molecule.

The third peptide investigated is termed Peptide CB7. This peptide, at present, has been only poorly resolved. However, some information has been obtained indicating that it is approximately 580 A long, has at least 5 separable less dense bands, and makes up

nearly 18% of the total SLS molecule. Placement of this peptide along the parent SLS molecule is somewhat questionable. However, initial observations suggest that Peptide CB7 is probably adjacent to Peptide CB6.

Since the three peptides, OB6, CB7 and DB8 comprise approximately 67% of the parent SLS collagen molecule, the other 5 known peptides present in collagen should be located within the remaining 33% of the molecule.

Significance to Dental Research

The knowledge that complement utilization is mediated by a particular substrate may eventually lead to a fuller comprehension of serum-cell interactions. This may include certain host reactions to endotoxic LPS which are initiated via the complement system. Continuation of these studies may lead to a further understanding of fundamental problems relating to oral microbiota and various pathological conditions.

Collagen is an important component of all connective tissues. Knowledge concerning its structural morphology in relation to its chemical composition would contribute considerably to an understanding of many normal and pathological processes.

Proposed Course of Projects:

1. Future work in this area will be basically a continuation of the present study. This will include an attempt to separate the various lipids present in the LPS and to determine which, if any, is responsible for the reaction between serum complement and endotoxic LPS. Other endotoxins and membranes will be examined in a similar manner.

2. Work is continuing in collaboration with the Laboratory of Biochemistry, NIDR, in an effort to morphologically map the collagen molecule with respect to known peptide sequences. Future studies may involve investigation of pathological collagen conditions with respect to possible variations in collagen structure at the peptide level.

Part B

Publications:

1. Mergenhagen, S. E., Gewurz, H., Bladen, H. A., Nowotny, A., Kasai, N. and Luderitz, O. Interactions of the complement system with endotoxins from A Salmonella Minnesota mutant deficient in O-polysaccharide and heptore. J. Immunol. 100:1, 1968.

Serial No. NIDR-44 (66)
1. Histology and Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: The Relationship Between Function and Structure in Microorganisms

Previous Serial Number: NIDR-23

Principal Investigator: Dr. G. J. Hageage

Other Investigators: Dr. H. A. Bladen, Dr. E. D. Eanes

Cooperating Units: Dr. R. Gherna (American Type Culture Collection); Dr. R. Steere (USDA)

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives:

The studies involving structure-function relationships under investigation in this laboratory have been concentrated in the area of membrane systems. Generally, the problems involve the morphological and chemical structure, the nature of the enzymes, and the functions associated with the total membrane fraction of gram negative bacteria. Specific objectives of the present study are three fold:

(1) To determine the site of sulfide oxidation in gram-negative bacteria

(2) To determine what role, if any, the cytoplasmic membrane plays in the oxidation of sulfides, and

(3) To determine the nature of the sulfur deposits derived from the oxidation of sulfides.

Methods Employed:

Chromatium and Thiospirillum species were grown in the laboratory of Dr. R. Gherna in screw top bottles containing a synthetic mineral medium, Vitamin B_{12}, and sodium sulfide. The bottles were illuminated with continuous light from a 25 Watt bulb.

Cells to be examined by electron microscopy were either negatively stained with phosphotungstic acid or were fixed, dehydrated, and embedded in Vestopal W according to the procedure of Kellenberger. Thin sections were cut and stained with uranyl acetate and lead citrate.

Autoradiography for electron microscopy were performed on cells grown in the presence of Na_2S^{35}. Grids containing thin sections were attached to glass slides, looped after the procedure of Caro with Ilford L-4 emulsion and stored in the dark for 2 weeks. For development the grids were immersed in Microdol-X for four minutes at 20°; washed in distilled water, fixed in an acid fixer for four minutes and finally washed in two changes of distilled water. Lead citrate was used to remove the gelatin as well as stain the sections.

Cells to be freeze-etched were glycerinated in 10% glycerin for 8 hours prior to freezing. Samples were placed on copper rivets and frozen in liquid freon 22. Following this treatment the specimens were fractured under liquid nitrogen in a vacuum evaporator with a pre-cooled scalpel. After etching at -100°C for one minute the specimens were shadowed with carbon-platinum and backed with carbon. The organic matter was digested with chromic acid and the fragments of replica rinsed in distilled water prior to being picked up on formvar-carbon backed grids.

The sulfur globules were isolated from Chromatium cells by osmotic lysis of spheroplasts prepared by lysozyme-EDTA treatment. The sulfur globules were collected by differential centrifugation and washed several times with distilled water. X-ray diffraction patterns of freshly isolated, wet sulfur packed in a 0.5 mm capillary tubes were taken with a 57.3 mm diameter powder camera at exposure times of one hour. Individual samples were then dried by vacuum for 30 minutes without removing the capillaries from their brass mounting rods. Consequently it was possible to examine approximately the same cross-sectional area of the same sample in both the wet and dry state.

Major Findings:

Where Chromatium or Thiospirillum cells devoid of sulfur inclusions were exposed to hydrogen sulfide and incubated in the presence of light they immediately began to accumulate round, refractile inclusion which increased in size until they occupied most of the cell volume. This event took 10-20 minutes. Electron autoradiography of cells exposed to

Na_2S^{35} revealed developed silver grains on or in close proximity to these inclusions. When viewed through crossed Nicol prisms, the sulfur inclusions, whether in the cell or isolated in a pure, wet state, had a characteristic maltese cross appearance. Rotation of the mount did not change the orientation of the arms possibly suggesting a symmetrical radial arrangement of the birefringent units. Drying of isolated sulfur inclusions resulted in the formation of birefringent crystals.

X-ray patterns of isolated wet sulfur inclusions gave two broad and diffuse diffraction rings with maxima at 3.75 Å and 4.84 Å. This pattern closely resembles the diffraction pattern of liquid sulfur. When dried, the same sample gave diffraction lines that agree with those of Orthorhombic sulfur as given in the ASTM powder diffraction file. In addition five diffraction lines (d= 5.98, 5.35, 4.55, 3.70, 2.96) were observed which could not be indexed as orthorhombic, rhombohedral or monoclinic sulfur.

Thin sections revealed that the cell envelope of Chromatium, like that of other gram-negative organisms, consisted of a highly convoluted outer membrane-like structure overlying a thin electron dense layer. The cytoplasm contained a membrane system which appeared as connected vesicles and bulged tubes. Work performed in other laboratories has shown that these structures (chromatophores) originate as tubular or vesicular invaginations of the cytoplasmic membrane. In freshly fed cells the greater part of the cytoplasm was occupied by sulfur inclusions. These inclusions, encompassed by an electron-dense border, had a density similar to that of the cytoplasm. In sections heavily stained with lead, cords of slightly denser material appeared to radiate from the center of the inclusions forming a stellate pattern.

Freeze-etched preparations revealed that the sulfur inclusions were enclosed not only by the border seen in thin sections but also by the same membrane which invaginates to form the chromatophores. This indicated that (1) the inclusions are not intracellular per se but are actually outside the cytoplasm, and (2) the enzyme(s) for the oxidation of sulfides reside on the cytoplasmic membrane. The latter conclusion is supported biochemically by the formation and accumulation of sulfur globules around Chromatium spheroplast and isolated membrane fragments after exposure of these entities to hydrogen sulfide.

Significance to Dental Research:

Studies concerning the relationship between structure and function, especially as concerned with the biologically universal "cell membrane" may lead to a further understanding of the fundamental problems relating to oral microbiota and various pathologic conditions.

Proposed Course of Project:

Studies concerned with the role of the cytoplasmic membrane in the oxidation of hydrogen sulfide and the nature of the sulfur formed will be continued.

Part B

Publications:

1. Hageage, G. J. Observations on the fine structure and cell surface of flexibacter species. Bacteriological Proceedings 1967. P. 25.

2. Doetsch, R. N. and Hageage, G. J. Motility in procaryotic organisms: problems, points of view, and perspectives. Biological Reviews. In press.

Serial No. NIDR-45 (67)
1. Histology and Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Electron Microscopic Radioautography: Response Characteristics of Nuclear Emulsion to High Energy Beta Particle Irradiation

Previous Serial Number: None

Principal Investigator: Dr. Robert C. Thompson

Other Investigators: None

Cooperating Units: IBM 360/50 Computing Facility, DCRT, NIH

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

Numerous investigators have stressed the importance of beta particle energy and range in relation to the resolution and sensitivity characteristics of electron microscopic radioautography. In particular it has been pointed out that the predicted density distribution of developed emulsion grains about a point source of radioactivity should follow a $Cos^3 \theta$ curve for very low energy particles, and a $Cos^2 \theta$ function for high energy isotopes. Extensive data exists for tritium confirming the predicted $Cos^3 \theta$ distribution, but limited data available from high energy studies using phosphorous-32 show a much broader distribution than that predicted.

It is probable that characteristics of the emulsion itself are responsible for the observed discrepancies, and this project concerns the experimental measurement of the response of nuclear emulsion monolayers to high energy beta particle irradiation.

Methods Employed:

Ilford L.4 nuclear emulsion was dissolved in water at 50°C and applied to carbonized Formvar coated grids using a standard looping technique described in the literature. Representative randomly chosen grids were observed with the electron microscope to confirm a monolayer grain distribution.

A point deposit of phosphorus -32 on the surface of a thin polypropylene film was mounted at the center of a large metal bell jar and emulsion coated grids were positioned a short distance away oriented at various angles to the direct path line from the source. After evacuation of the chamber the source was uncovered for the desired irradiation time period using a remote control operated motor to appropriately position a lead shield. Control grids in the chamber protected from direct rays were used to assay scattered background radiation, and other grids completely protected from beta radiation by a heavy clear glass container served to control emulsion background and stray light exposure.

Following irradiation the grids were developed, fixed, and dried using a standardized procedure, and were then observed with the electron microscope. Grain counting was performed over randomly chosen fields of each grid and the results analyzed using standard statistical techniques.

Certain mathematical computations of this study were of necessity executed on the digital computer.

Major Findings:

Very high energy beta particles passing through thin films are known to suffer negligible fractional energy loss and little or no scattering in direction; under such conditions the number of exposed grains produced per unit of particle path length is a constant, and this is an underlying assumption of the $\text{Cos}^2\ \theta$ distribution derivation. Such a particle striking a uniform emulsion layer at an angle of θ from the normal is expected to yield secant θ times the number of exposed grains produced by the particle incident normally.

However, the results of our experimental studies using phosphorus -32 clearly showed a stronger dependency on irradiation angle than secant θ for certain angles; sensitivity values at 35° incidence and 70° incidence were respectively 1.9 and 1.3 times higher than predicted based on the above theories ($P=0.02$). It was concluded that scattering events are probably responsible for the increased sensitivity and that these effects begin to become important at emulsion path lengths as short as 2000 A for particles from phosphous -32.

A new predicted curve was constructed by plotting the product of $\cos^2 \theta$ and the corresponding sensitivity ratio for that θ value against tangent θ. The resulting curve was found to match the broad shape of the published experimental data referred to earlier in this report.

It was concluded that modification of the theoretical distribution curve to include the influence of angle of incidence on emulsion sensitivity as measured in these studies will largely eliminate the discrepancies between theory and certain experiments noted previously in the literature.

A paper concerning these findings is in preparation.

Significance to Dental Research:

Extension of the system of electron microscopic radioautography to include bone seeking isotopes of high energy is currently of great interest and knowledge of the practical resolution and sensitivity characteristics of the system under these conditions is required for valid data interpretation.

Proposed Course of Project:

This investigator's tenure of appointment ceases on June 30, 1968. This work will be continued at another institution.

Part B: not included

225

Serial No. NIDR-46 (66)
1. Histology & Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Uranium Tracer System Employing Nuclear Fission Reaction

Previous Serial No: NIDR-24

Principal Investigator: Dr. Robert C. Thompson

Other Investigators: None

Cooperating Units: Nuclear Reactor Facility of the Armed Forces Radiobiology Research Institute, Bethesda, Md.

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description

Objectives:

A previous report of this project described the basic characteristics of tracks in various thin films resulting from fission of uranium atoms deposited on the films.

The purpose of current studies of this high resolution tracer system is threefold: a) to refine and standardize experimental methods of producing registration films; b) to investigate uranyl ion-calcium ion exchange sites on the surface of synthetic hydroxy apatite crystals, and c) to investigate uranium deposition in vivo in long bones of rats given injections of uranium.

Methods Employed:

Electron microscope grids covered with collodion or Formvar films were coated with thin evaporated films of chromium, silicon monoxide, or boron carbide in different experiments. Chromium films were produced by evaporation from a tungsten wire loop previously electroplated with a known weight of chromium. Careful evaporation of a known weight of

silicon monoxide from a tungsten wire basket was found to be suitable for producing films of this material. Boron carbide was evaporated from a cavity in a carbon rod heated by passing very high current through the rod, or from a special carbon arc constructed with a hollow center in one carbon rod packed with boron carbide. Structural analysis of the evaporated films was attempted using x-ray and electron diffraction techniques.

Synthetic hydroxy apatite was prepared by a precipitation reaction of calcium nitrate and ammonium phosphate carried out at pH 10 and in a CO_2 free environment. The precipitate was allowed to mature 3 days at 100°C and pH 10. After washing with distilled water the product was stored wet for several months prior to use. X-ray diffraction, electron microscopy, and infrared spectroscopy analyses were consistent with an hydroxy apatite of low carbon dioxide content and small crystal size.

Exchange reactions were carried out for 15 minutes at room temperature in filtered aqueous solutions of uranyl acetate containing either natural uranium or enriched U-235.

Using centrifugation, the exchanged hydroxy apatite was washed successively in water, twice with acetone, and isoamyl acetate, and suspended in a final solution of 20% collodion in isoamyl acetate. Thin films of this material prepared by the casting on water technique were found to provide satisfactory dispersion and fixation of the crystals on the electron microscope grids.

For the short term animal studies rats were given pharmacologic doses of uranyl or sodium acetate and were sacrificed 3 hours later. Both femurs and tibiae were cleaned and immediately freeze-dried. Small portions of the shaft of the bones were embedded directly and thin sections taken for electron microscopy.

Grids containing the samples were irradiated in the AFFRI nuclear reactor facility and following a suitable cooling-off period were observed with the electron microscope.

Major Findings:

Thin collodion membranes without additional evaporated supporting films are unstable and are destroyed in the nuclear reactor. Light carbon evaporated onto such films provides only minimal protection, but in areas of membrane that do happen to survive irradiation, tracks appear as extremely wide linear gaps in the membrane. Carbonized formvar membranes were found to be more durable. Silicon monoxide films are highly membrane protective, but also appear to obscure or reduce the damage to the membrane so that tracks are difficult to distinguish from general background. Boron carbide or boron-carbon films if thick tend to "decorate" tracks with dark material located along

the axis. Lighter films of the same material appear to produce a stippling effect of separate black dots throughout the area of the track. Chromium films provide good membrane protection and also produce a narrower track of higher contrast. All types of films can completely obscure any tracks if too thick a layer is used.

X-ray and electron diffraction studies of boron containing films reveal a predominantly amorphous pattern; only when the material evaporated was highly crystaline could any consistent lines be obtained from the resulting films. It is believed that films produced by evaporating powdered boron carbide or a boron-graphite mixture from a carbon rod are probably a mixture of amorphous boron carbide and carbon.

Tracks associated with hydroxy apatite crystals from a uranium exchange reaction were seen only rarely, and none were seen in areas devoid of visible crystals. The limited number of tracks prohibited any conclusions about the distribution of the uranium. No change in crystal morphology was noted, and therefore one could elect to irradiate for a longer period to produce more tracks. Alternatively the use in a current experiment of enriched uranium-235 uranyl acetate is expected to yield 130 times as many tracks for the same amount of material and duration of irradiation.

Bone tissue density and background variation seemed to be responsible for the failure to observe tracks in these sections; occasional holes in the membranes and section cannot clearly be called tracks. Distinct morphological differences were apparent between sections from experimental and control animals, but no visible areas of increased electron density suggestive of uranium deposition were noted.

Significance to Dental Research

The development of this tracer system using fissionable "bone seeking" elements may provide a useful new method of extremely high resolution for ultrastructural studies of the mineralization process and surface exchange reactions of mineral crystals.

Proposed Course of Project:

The investigator's tenure of appointment ceases on June 30, 1968. This work will be continued at another institution.

Part B: not included

Serial No. NIDR-47 (55)
1. Histology and Pathology
2. Histochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Studies of Human Collagenase

Previous Serial Number: NIDR-25

Principal Investigator: Dr. H. M. Fullmer

Other Investigators: Dr. W. A. Gibson, Dr. J. F. Goggins, Dr. G. S. Lazarus

Man Years:

Total:	2 1/4
Professional:	1 3/4
Other:	1/2

Project Description:

Objectives:

A. To produce collagenase by cultures of human gingivae in sufficient quantities to permit isolation and purification. The purified enzyme can be used for a number of studies including (a) if antigenic, for the production of antisera which can be conjugated with a fluorescent tag to be employed for the determination of the cell types producing the enzyme (b) for the characterization of the action of collagenase on the collagen molecule, (c) for characterization of optimal conditions of enzymatic activity as well as determination of its inhibitors and activators.

B. To determine the amount of collagenase detectable from (a) certain body fluids, particularly spinal fluid, urine and blood, and (b) cultures of certain tissues, particularly skin, bone synovia, brain, spinal cord and muscle and the relationship of these amounts to certain connective tissue and neuromuscular diseases such as amyotrophic lateral sclerosis, scleroderma, rheumatoid arthritis, etc.

Methods Employed:

1. Tissue culture: Gingival samples, excised in the course of treatment of various diseases, are provided by local dentists. After cleansing and mincing, the specimens are cultured in Tyrode's solution with antibiotics at 37°C in a CO_2 incubator supplying 5% CO_2 in air. Culture fluid is collected and replenished every 2 days for three harvests. Cells and sediments are separated from culture fluids by centrifugation at 18,000 rpm. Collagenase is obtained by purification of the culture fluid.

Various other tissues are obtained from surgery or at autopsy. They are likewise cleansed, minced into small pieces, and cultured. Culture fluids are assayed for collagenase.

2. Separation methods: Culture fluids have been submitted to electrophoresis on the Brinkman Model FF apparatus. Good separation has been achieved utilizing tris buffer pH 8 with 0.12 M on the electrode and 0.04 M on the curtain. Column chromatography utilizing Sephadex G200 has provided further separation. Use of acrylamide gels aids determination of the degree of purification achieved during separation procedures.

3. Collagenase assay methods:

A. Gel inhibition: Collagen solutions brought to 37°C form a gel. Addition of collagenase to the collagen solution prevents gelation, and the degree of prevention of gelation is a function of the amount present per unit of time. The degree of prevention of gelation is a function of the amount present per unit of time. The degree of prevention of gelation is read in a Klett photometer as units of opacity. This method has frequently been found to be unreliable.

B. Viscometry: Collagen solutions manifest a characteristic viscosity at 20°C. Addition of collagenase to collagen solutions results in a reduction of viscosity measured in time.

C. Release of radioactive degradation products: ^{14}C-glycine has been administered to young growing rats that incorporate the isotope into collagen. The collagen is extracted, purified and used as a substrate for collagenase. The release of radioactive degradation products from purified collagen per unit of time is a measure of collagenase activity. A liquid scintillation counter is utilized.

Major Findings:

In 1965 we demonstrated the existence of collagenase in man. Prior to this, collagenase had been detected only in cultures of certain microorganisms, and of tadpole tails--particularly during the process of metamorphosis. Our observations indicated that cultures of gingivae that had been excised for the treatment of periodontal diseases produce a collagenase.

Collagenase has now een detected in cultures of normal human skin, and increased amounts were detected in culture fluids of skins taken from individuals with certain neuromuscular diseases; namely, amyotrophic lateral sclerosis, progressive spinal muscular atrophy, myotonic dystrophy, occulopharyngeal neuromuscular disease, Parkinsonism periodic paralysis, myasthenia gravis and polymyositis. These findings are in accord with our 1960 observations which reported the existence of a connective tissue disorder, demonstrable morphologically and histochemically, in the dermis of 60% of individuals with amyotrophic lateral sclerosis. Collagenase was also found in the culture fluids of skins taken from individuals with scleroderma, dermatomyositis, and certain other connective tissue diseases provided the individual was not on prednisone or prednisilone therapy. Our data is consistent with the hypothesis that the collagenase detected is the one concerned with the normal metabolic turnover of collagen, and that something happens during certain diseases which permits detection of increased amounts of the enzyme in cultures of skin.

Collagenase has been detected in culture fluids of articular and growth plate cartilates of man, goat and rabbit and in culture fluids of vertebrae, mandible, maxilla and pieces from long bones of man and goat.

Data from acrylamide gels and electron microscopy indicates that the collagenase from gingivae, bone, and granules of leukocytes acts on the collagen molecule in a fashion identical to the collagenase derived from tadpoles; that is, the collagen molecule is cleaved only once resulting in a 3/4 piece and a 1/4 piece. Subsequently, other enzymes in culture fluids and in the granules of leukocytes act on the cleaved collagen molecules resulting in numerous digestion products.

preliminary data indicates human collagenase has a broad range of activity from pH 7-9, and scant activity below pH 7. It is completely inhibited by EDTA, and partially by cysteine. It has an absolute requirement for calcium in low concentrations and is inhibited by high concentrations of calcium. It has the capacity to reduce the viscosity of 0.20% solutions of collagen as much as 35-55% in 20 hours at 20°C as compared to 2% for control solutions.

Significance to Dental Research:

The consequence of periodontal diseases is the loss of periodontal fibers, principally collagen, and bone of which collagen is the major organic constituent. It is difficult to conceive of any oral disease in which collagen, the substrate of collagenase, is not involved. We have a system which is able to assay the catabolism of this very important protein. Utilization of this system will provide greater understanding of connective tissues in health and disease.

Proposed Course of Project:

The major effort will be directed toward the production, purification and characterization of collagenase. Further attempts will also be made toward elucidation of the relationship of detectability of collagenase in cultures of skin, bones, synovial membranes, brains, spinal cords and muscles to neuromuscular and other diseases.

Part B:

Publications:

1. Fullmer, H. M. The development of oxytalan fibers. "Mechanisms of tooth support" A Symposium Oxford 6-8 July, 1965. John Wright and Sons, 1967, pp 72-75.

2. Fullmer, H. M. A decalcification technic for enzyme preservation. "Symposium on the dental pulp" J. Dent. Res. (In press).

3. Fullmer, H. M., Lazarus, G. S., Stam, A. C., Gibson, W. A. Collagenase in neuromuscular disease. "First National Symposium on amyotrophic lateral sclerosis" 1967. (In press).

4. Fullmer, H. M., Lazarus, G. S. Collagenase in human, goat and rat bone. Israel J. Med. Sci. 3:758-761, 1967.

Serial No. NIDR-48 (64)
1. Histology and Pathology
2. Histochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Histochemical and Chemical Studies of Connective Tissues

Previous Serial Number: NIDR-26

Principal Investigator: Dr. W. A. Gibson

Other Investigators: Dr. H. Fullmer, Dr. J. Goggins

Cooperating Units:

Man Years:

Total:	2 3/4
Professional:	2 1/4
Other:	1/2

Project Description:

Objectives:

A. To assay various histochemically detectable substances in normal and diseased tissues through the use of existing techniques, by modification of existing techniques, or through the development of new techniques.

B. To determine various metabolic parameters of normal and diseased tissues, and to use and develop techniques for the detection and assay of the factors involved.

C. To develop defined in vitro systems of tissues and cells in continuous as well as primary cultures and to utilize such systems for histochemical and biochemical studies of normal and diseased tissues.

Methods Employed:

The cells and tissues utilized in these studies include those from oral and other regions obtained from human and animal sources.

A. Histochemical:

Since enzymes are the substances of interest in the current studies, tissue specimens are handled in a manner to minimize the loss of enzyme activity. This includes freezing and sectioning in a cryostat. Prior demineralization of mineralized tissues is accomplished by a process devised by Balogh, and Fullmer and Link. The resulting sections are subjected to various staining procedures to determine the site and quantity of particular enzymes.

Modification of existing techniques and the development of new techniques involve qualitative and quantitative changes in substrates, cofactors, dyes, activators and inhibitors and conditions such as pH and temperature.

B. Biochemical:

Quantitative assays of several selected enzymes are being performed to evaluate the effects of tissue processing, especially the demineralization procedure currently employed on the accuracy and reliability of the histochemical methods.

Electrophoretic separations and identification of various enzymes and their molecular variants are being performed to supplement information from histochemical studies as well as to evaluate histochemical methods.

C. Cell and Tissue Culture:

Standard and original techniques are being utilized in current studies of various cells and tissues in primary culture.

Quantitative studies of primary human and animal oral cells grown in vitro have been hindered by the lack of methods of growing large numbers of uniform cultures partially because of damaging disaggregation technics used. Classical technics such as trypsin disaggregation have proven useless because of cell injury. Technics utilizing other enzymes such as collagenase, elastase and hyaluronidase are being tested.

Major Findings:

A. The continued histochemical study of the periodontal ligament and alveolar bone has revealed widespread and impressive amounts of various enzymes. Non specific esterase activity was correlated with the cellular activity associated with active remodeling. Osteoclasts stained intensely in all sections while the staining of osteoblasts and cementoblasts ranged from little or none in quiescent areas to intense in areas of active bone and cementum formation respectively. Fibroblasts stained most intensely in areas of active remodeling.

In sections stained for cholinesterase activity, Schwann cells, red blood cells, megakaryocytes and muscle motor end plates stained intensely. True acetyl-cholinesterase activity was demonstrated in red blood cells, megakaryocytes and muscle motor end plates, but no nerve associated true acetylcholinesterase was detected in the rat periodontal ligament.

In sections stained for a number of selected glycosidases, osteoclasts stained intensely for β-glucuronidase, β-N-acetylglucosaminidase, β-galactosidase and β-blucosidase. Osteoblasts and cementoblasts in areas of bone and cementum formation as well as fibroblasts, superficial osteocytes and cementocytes in areas of active remodeling were similarly but less intensely stained for the same enzymes. Little or no staining occurred for any of the enzymes studied in cells located in more quiescent areas.

B. Zymograms of the rat jaw esterases consistently demonstrated 6 major bands of activity. When 10^{-5}M eserine sulfate was incorporated into the incubation media the total esterase activity was diminished only slightly. However, band number 4 was reduced to a trace. This confirms the histochemical finding that most of the esterase activity of the rat jaw is non-specific in nature. When 10^{-5}M E-600 was incorporated into the incubation media all but band number 2 and a portion of band number 3 were eliminated. This confirms the histochemical finding that the bulk of the non-specific esterase activity is E-600 sensitive or B type esterase, while a small but definite amount of E-600 resistant or C type esterase is present in the rat jaw tissue.

C. A technique devised by M. Neiders for growing human gingival epithelial cells in spinner culture is being adapted in this laboratory for routine plate cultures. The isolation of the epithelial cells is obtained by first separating the epithelium from the dermis by elastase treatment. The cells are then dispersed by treatment with solutions containing low concentrations of trypsin: the yields of uniform viable cells are greater than that obtained with any other method used to date.

Significance to Dental Research:

The overall objectives of the project are designed to lead to a further understanding of the physiologic and pathologic processes occurring in oral tissues.

Proposed Course of Project:

Histochemical, biochemical, and cell and tissue culture investigations of connective tissues will continue. The areas of specific interest will be the metabolic analysis of normal and diseased tissues as revealed by qualitative and quantitative enzyme histochemistry. The modification of techniques and the development of new techniques and tools of research will be no small part of the developing program of research.

Part B

Publications:

1. Gibson, W. A. and Fullmer, H. M.: Demonstration of 5'-nucleotidase activity in decalcified bones and teeth. J. Histochem. Cytochem. 14:934-935, 1967.

2. Gibson, W. A. and Fullmer, H. M.: Histochemistry of the periodontal ligament: II. The phosphatases. Periodontics 5:226-232, 1967.

3. Gibson, W. A. and Fullmer, H. M.: Histochemistry of the periodontal ligament: III. The esterases. Periodontics. 6:71-77, 1968.

Serial No. NIDR-49 (66)
1. Histology and Pathology
2. Histochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Histochemical and Chemical Studies of Connective Tissues and Teeth

Previous Serial Number: NIDR-27

Principal Investigator: Dr. J. F. Goggins

Other Investigators: Dr. H. M. Fullmer, Dr. W. A. Gibson, Dr. G. S. Lazarus

Cooperating Units: None

Man Years:

Total:	1 1/4
Professional:	1
Other:	1/4

Project Description:

Objectives:

A. To determine various metabolic parameters of normal and diseased tissues and to use and develop techniques for the detection and assay of the factors involved.

B. To relate these factors to the tissue physiology of pathology by the development and application of qualitative and quantitative histochemical and biochemical methods.

C. To determine the feasibility of preserving viability and enzyme activity in oral tissues after freezing and prolonged storage.

Methods Employed:

A. Histochemical:

Since enzymes are the substances of current interest tissue specimens are handled in a manner to minimize the loss of enzyme activity. This includes freezing and sectioning in a cryostat. The

resulting sections are subjected to various staining procedures to determine the site of particular enzymes. Enzyme activity is related to cell type, function, age, differentiation, etc.

B. Biochemical:

Hyaluronidase activity in human gingival tissues and rab it alveolar macrophages is being investigated. The ability of various tissue preparations to degrade hyaluronic acid is assayed by determination of the release of N-acetylglucosamine reactive groups by the method of Reissig, Strominger and Leloir. The method has been adapted for micro determinations. The effects of pH, enzyme concentration and other factors on hyaluronidase activity are being studied. Products of hyaluronic acid released by hyaluronidase action are being isolated with the aid of ion exchange chromatography.

C. Cryobiological:

Existing methods of controlled freezing have been applied to oral tissues in conjunction with the use of protective agents. After storage for various periods of time the tissues are cultured in vitro to determine viability. Some are analyzed histochemically.

Major Findings:

A. Hyaluronidase has been detected in isolated alveolar macrophages. The enzyme manifests a pH optimum between 3.9 and 4.2 as does gingival hyaluronidase. No activity was detected above pH 5. With increasing time, progressively smaller oligosaccharides were detected. Several bacterial hyaluronidases were inactive under conditions of the assay.

B. Experiments indicate that human gingival tissue can be successfully frozen and stored with preservation of enzyme activity and viable cells for up to at least 14 months. Viability was demonstrated by cell outgrowth. Histochemically, enzyme activity was demonstrated for 10 oxidative and 3 hydrolytic enzymes after 14 months storage. The distribution was similar to that of fresh frozen tissue and enzyme activity was demonstrable within incubation periods usual for fresh-frozen tissue.

Significance to Dental Research:

The overall objectives of the histochemical and biochemical enzyme studies are to lead to a better understanding of the physiological processes occurring in oral tissues and to relate these to any changes found in pathological conditions.

Proposed Course of Project:

Histochemical and biochemical investigations of enzymes in connective tissues will continue. A particular field of study will be the correlation of data from utilization of the Lowry quantitative microchemical methods with that from histochemical methods to obtain reliable information about the metabolic status of small regions. Qualitative assays suggest wide variations of enzyme activity in various regions of periodontal membranes, for example, depending on the function of the cells at any particular time.

Part B

Publications:

1. Goggins, J. F. and Fullmer, H. M., Hydrolytic enzyme histochemistry of the rat molar pulp. Arch. oral Biol., 12:639-644, 1967.

2. Goggins, J. F., Fullmer, H. M. and Steffek, A. J., Hyaluronidase activity of human gingiva. Arch. Path., 85:272-274, 1968.

3. Goggins, J. F. and Gibson, W. A., Histochemistry of viable frozen-stored human gingiva. J. Invest. Derm., (In press).

Serial No. NIDR-50 (67)
1. Histology and Pathology
2. Histochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Collagenase Activity of Human Normal and Diseased Tissues

Previous Serial Number: None

Principal Investigator: Dr. Gerald S. Lazarus

Other Investigators: Dr. H. M. Fullmer, Dr. John Daniels
Dr. J. F. Goggins, Dr. H. A. Bladen

Cooperating Units: Dr. John Decker, NIAMD; Dr. Robert Brown, NCI; Dr. Cap Oliver, GWU Hospital; Dr. Carter Multz, WRAMC; Dr. Werner Barth, NIAMD

Man Years:

Total:	2 1/4
Professional:	1
Other:	1 1/4

Project Description:

A. To purify, characterize and elucidate the action of collagenase on the collagen molecule.

B. To study the role of collagenase activity in synovia of patients with rheumatoid arthritis, and to attempt characterization of the pathophysiology of rheumatoid arthritis.

C. To define the role of granulocyte collagenase in inflammation.

Methods Employed:

A. Biochemical

Human granulocytes were extracted and the collagenase purified by ion exchange chromatography on DEAE. The enzyme was characterized by viscometry, acrylamide gel electrophoresis, polarimetry and radioactive

reconstituted collagen fibril assays. In addition, inhibitors and activators of collagenase activity were studied using previously described technics. Similar technics were employed with rheumatoid collagenase.

B. Morphological

The morphology of segment long spacing collagen (SLS) which has been digested with collagenase has been studied by electron microscopy.

Major Findings:

A specific collagenase was found in the granule fraction of human granulocytes. The enzyme cleaves the collagen molecule into two discrete products. These pieces, representing 3/4 and 1/4 of the molecule appear identical to those produced by tadpole and synovial collagenases. The enzyme is specifically inhibited by glutathione and cysteine.

Increased collagenase activity was found in cultures of synovia of patients with rheumatoid activity. The amount of collagenase detected correlates with the clinical severity of the disease. This suggests that collagenase activity may play a significant role in the disease process.

Significance to Dental Research:

An understanding of collagenase metabolism gained from the granulocyte and synovial studies will increase our appreciation of pathophysiology of connective tissues everywhere. In addition, technics utilized are being applied to collagenase derived from other sources.

Proposed Course of Project:

Collagenase studies will continue within this section and in Boston where I begin July 1, 1968.

Part B

Publications:

1. Lazarus, G. S. and Mowry, F. M. Endocarditis localized to the tricuspid valve: report of a case. Michigan Medical Center Journal. 33:219, 1967.

2. McDonald, F. D., Lazarus, G. S., and Campbell, W. L. Phenylbutazone anuria: Southern Medical Journal 60:1318, 1967.

3. Lazarus, G. S., Brown, R. B., Daniels, J. D., and Fullmer, H. M. Human granulocyte collagenase. Science 159:1483, 1968.

4. Brown, R. B., Lazarus, G. S., and Fullmer, H. M. Collagenolotic activity of human granulocytes. Blood. 30:856, 1967.

5. Lazarus, G. S., Daniels, J. D., Brown, R. B., Bladen, H., and Fullmer, H. M. Mechanism of destruction of collagen by human white cells. Journal of Clinical Investigation. (In press).

Serial No. NIDR-51 (67)
1. Histology and Pathology
2. Crystal Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: X-ray Diffraction Studies on Fibrous Proteins

Previous Serial Number: None

Principal Investigator: Dr. E. D. Eanes

Other Investigators: Dr. E. J. Miller

Cooperating Units: Dr. G. G. Glenner, NIAMD-LEP

Man Years:

Total:	2 3/4
Professional:	1 1/2
Other	1 1/4

Project Description:

Objectives:

The continuing objective of this project is to study the x-ray diffraction properties of fibrous proteins and to relate these properties to structural parameters of interest to hard tissue biology. During the year covered by this report, two studies were undertaken with the following more specific objectives in mind:

1. To compare the crystallographic properties of bone and soft tissue collagens under a variety of experimental situations with specific intent of establishing the relationship between structural stability in these collagens and the degree of covalent cross-linking.

2. To determine the crystallographic features underlying the structure of the fibrous protein component of amyloid tissue.

Methods Employed:

The principal technique employed in both studies was wide-angle x-ray diffraction. Collagenous material suitable for diffraction analysis was obtained from demineralized tibia of 3-week-old chicks and from

the tail tendon of rats. Suitably cut segments were mounted in glass capillaries and x-ray diffraction diagrams taken with a Chesley-Philips microcamera. The collagen segments were examined either dried or in contact with one of the following test solutions: distilled H_2O, 0.5M acetic acid, 5M guanidine hydrochloride.

Amyloid tissue was obtained from the organs of human patients and experimental animals afflicted with amyloidosis. Both fresh tissue and purified fibrous extracts were studied. The material was examined by essentially the same diffraction procedures as employed in the collagen study as well as with standard powder diffractometry techniques.

Major Findings:

1. Collagen study: The diffraction patterns from dried tendon and dried bone collagen were alike in all fundamental respects except the arcing of the diffracting lines from the bone collagen was more extensive. This finding demonstrates that the monomeric units of these two collagens have the same basic crystallographic properties but that the alignment of the fibers at a tissue level of organization is less ordered in the bone collagen than in the tendon.

The diffraction data further demonstrated that the two tissue collagens were also alike in their response to the test solutions. In H_2O and 0.5M acetic acid, no breakdown in intramolecular triple helical structure took place for either collagen, even though extensive (>5Å) lateral separation of adjacent molecular units did occur. When in contact with 5M guanidine, on the other hand, both collagens became completely denatured.

Differences in diffraction properties reflecting important distinctions in the two collagens at an intermolecular level of structural organization did not become evident until attempts were made to re-nature guanidine denatured material. When washed free of guanidine and dried, the diffraction pattern of bone collagen was identical to that of undenatured material. In contrast, the tendon pattern following the same re-naturation procedure showed no signs of native collagen structure. These results indicate that bone collagen can elastically renature under conditions where the monomers of tendon collagen cannot reestablish their helical structure and lateral relationships. Native collagen structure was observed, however, in formalin-fixed rat tail tendon following subjection to the same denaturation-renaturation routine.

The above experimental findings can be interpreted in terms of the degree to which covalent cross-linking between adjacent monomers is known to occur in these collagens. In bone collagen, the presence of intermolecular cross-links prevent random dissociation of adjacent monomers during denaturation even though the individual polypeptide chains become randomly coiled. This preservation of interchain associations

apparently assures reassembly into native configurations upon renaturation. The absence of such cross-links in tendon collagen results in the loss of all spatial associations during denaturation with the consequence that upon attempted renaturation irregular non-helical associations form preferentially. Confirmation of this interpretation was found in the fact that covalently cross-linking tendon by formalin fixation enabled this collagen to become more like bone collagen in its ability to recover from guanidine denaturation.

2. Amyloid study: The position of the diffraction lines in unoriented x-ray patterns obtained from fibrous amyloid protein classified this material as of a β-type. The polypeptide chains giving rise to such patterns are in a pleated sheet configuration in which adjacent chains are in an antiparallel arrangement. Further, a cross-β pattern was obtained from samples in which fiber orientation had been induced by mechanical means. This latter finding suggests that the chain axes are directed perpendicular to the fiber direction. The β-type pattern was produced by all amyloid laden tissue studied which included material from mouse and duck as well as from human. In addition, the β-type pattern obtained from fresh wet tissue was unaltered by subsequent drying and purification. It appears, then, that the pleated sheet structure is a natural and stable configuration for amyloid protein fibers.

Significance to Dental Research:

1. Since collagen is an important component in all connective tissue including dentin, knowledge concerning the physical and chemical properties cross-links impart to this fibrous protein would further contribute to an understanding of its role in tissue structure and function.

2. The amyloid fiber and enamel matrix are the only naturally occurring cross-β proteins in human tissue reported to date. Studies on fibrous amyloid protein, therefore, may have particular relevance to dental research in that it may contribute to a more complete understanding of the nature and function of the protein matrix in enamel tissue.

Proposed Course of Project:

The wide-angle x-ray diffraction phase of these projects is essentially completed. Collagen, however, has a well developed pattern in the small-angle region of x-ray scatter. Studies will be conducted to explore the effect cross-linking may have on this small-angle diagram of collagen. The amyloid fiber also appears to scatter x-rays at small angles, and studies will be undertaken to accurately record these small-angle diffraction lines in the expectation of obtaining additional information on the structure of the amyloid fiber.

Part B: Not included

Serial No. NIDR-52 (63)
1. Histology & Pathology
2. Crystal Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Infrared Absorption Spectrophotometric and X-ray Diffraction Studies of the Inorganic Portion of Teeth and Bones and Related Synthetic Compounds

Previous Serial Number: NIDR-20

Principal Investigator: Mr. B. O. Fowler

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

The main objective is to define more clearly the composition of the inorganic phase of teeth and bones. The reactions of various synthetic calcium phosphates with different substances under varying experimental conditions are being determined and the results related to biological mineral systems. Additional objectives involve studies to ascertain the chemical bonding occurring between the organic and inorganic components in hard tissue.

Methods Employed:

The primary methods used are infrared spectroscopy and x-ray diffraction. Specialized infrared techniques involve utilizing reflectance, polarization, low and high temperature, and high pressure devices in obtaining spectra. In addition, physical-chemical techniques for synthesis and purification of hydroxyapatite, fluorapatite, carbonate apatite, and related calcium phosphates are employed. High temperature and pressure apparatus is designed and constructed in order to synthesize certain

substituted calcium phosphates in well-crystallized form, and study their reactions in vacuum or with carbon dioxide and/or steam under pressure at temperatures to 1100°C. The techniques are supplemented by chemical analyses to determine calcium, phosphorous, fluoride, carbonate, and trace elements.

Major Findings:

The OH stretching and librational infrared absorption bands of hydroxyfluorapatite (HFA) have previously been characterized and assigned. In order to obtain additional characterization, the HFA spectra have been examined down to 200 cm^{-1}, the frequency limit of the available instrumentation. One HFA band in this region was found to shift as a function of fluoride content. A study was made to establish the reason(s) for this band shift, in order to further characterize the structural features and infrared spectra of this biologically important compound.

The fundamental infrared absorption bands in hydroxyapatite (HA) arising from internal vibrations (stretching and bending modes) of the phosphate groups and the OH stretching and the OH librational modes have been given assignments. The OH stretching and librational modes have been assigned with certainty as based on shifts upon deuteration. The ν_3, ν_1, ν_4, and ν_2 phosphate bands have been primarily assigned on the basis of (a) the theoretically predicted bands arising from the spatial geometry of the phosphate group and (b) previously recorded Raman spectra of the phosphate group. The ν_3, ν_1, and ν_4 vibrational assignments of the phosphate group are most probably correct. The bands which have been assigned to the ν_2 vibration by several investigators is now questionable as a result of information obtained in the present studies which suggests a new assignment for the ν_2 band and other low-frequency bands.

Three major HA bands occur in the low-frequency region at 342,280 and 230 cm^{-1}. The band at 342 has a shoulder at about 355 cm^{-1} and the band at 280 cm^{-1} splits into a well-resolved doublet at liquid-nitrogen temperature. The major bands at 342 and 280 cm^{-1} have been assigned by several investigators to the ν_2 components of the phosphate groups. The band at 230 cm^{-1} has apparently not been reported.

Three approaches utilizing (a) band intensity, (b) isostructural analogues, and (c) isotopic substitution were employed to assign and/or reassign bands in the low-frequency region.

Band Intensity: the ν_1 and the ν_2 modes of the undistorted tetrahedral phosphate group are infrared inactive; however, these modes may become active when the phosphate group is distorted to lower symmetry. To establish the ν_1, ν_2 band intensity relationship compounds containing HPO_4 groups, for which both the ν_1 and ν_2 modes are active, were examined. A series of HPO_4 salts containing cations with increasing mass, $CaHPO_4$,

$SrHPO_4$ and $BaHPO_4$ was chosen in order that lattice vibrations would be shifted to lower frequencies and as a consequence not introduce interfering absorption in the ν_2 region.

Two major bands at 400 and 265 cm^{-1} were observed for $CaHPO_4$ in the 450-200cm^{-1} region; $SrHPO_4$ and $BaHPO_4$ each have only one band at about 415 cm^{-1}. The bands at 400 and 415 cm^{-1} are most probably the ν_2 components and the ν_2 band intensity is about one-half the intensity of the ν_1 band. By analogy the intensity of the ν_2 band in HA should be less than the intensity of ν_1. However, the intensity of the bands in HA which have been assigned to ν_2 previously are approximately twenty times the intensity of the ν_1 vibration in HA.

Isostructural Analogues: Since there are three major bands in the 400-200 cm^{-1} region of HA with two bands showing additional structure, the weak ν_2 bands, which would comprise only about one-twentieth of the area of these bands, could be superimposed on the major bands and therefore not easily detected. In order to shift interfering bands from this region, the isostructural analogues of HA, strontium apatite (SrA) and barium apatite (BaA) were prepared and examined in this region. Lattice vibrations arising from the isostructural analogues with heavier cations, i.e. Sr and Ba should show greater shifts to lower frequencies than the fundamental phosphate modes of these analogues.

SrA has two bands in this region at 322 and 235 cm^{-1}. The SrA band at 235 cm^{-1} is too low in frequency to be a component of ν_2. The intensity of the 322 cm^{-1} band is about four times the intensity of the ν_1 band and it shifts by about 10 cm^{-1} on deuteration. The intensity and shifts on deuteration indicate that this band is not ν_2 but rather arises from translational motion of the OH groups.

Only preliminary results are available on BaA in this region; however, only one definite band is observed in the 400-210 cm^{-1} region at 280 cm^{-1}. Both the intensity of this band, which is several times that of the ν_1 band and its low-frequency position indicate that this band is not ν_2.

The low-frequency bands in HA at 280 and 230 cm^{-1} are apparently lattice vibrations judging from the shifts of the corresponding bands in the Sr and Ba apatite analogues.

Isotopic Substitution: The bands in HA (342), HFA (355-345) and SrA (322 cm^{-1}), suspected of arising from the OH groups, were found to shift by approximately the expected amount on deuteration for translational motion of the OH groups. Attempts were made to further characterize these bands by mass dependancy utilizing O^{18}.

Samples were prepared by treating dehydrated samples of HA or other $CaO \cdot P_2O_5$ mixtures of the proper proportions with H_2O^{18} contained in sealed vycor tubes at pressures from 5 to 20 atmospheres in the

temperature range 500 to 1100°C. At 500°C apatite samples were prepared which contained approximately 40% of the total OH groups replaced by $O^{18}H$ groups; however, the samples were too poorly crystallized to definitely establish a shift in the 342 cm^{-1} band. Samples heated with H_2O^{18} (5 atm) at 500°C for five days not only showed $O^{18}H$ substitution, but in addition, the O^{18} from the water had exchanged with the oxygens of the orthophosphate groups. That O^{18} had exchanged with O^{16} in the phosphate groups was established by both new and shifted bands that appeared in the spectra of these samples.

This exchange was fortuitous, in that the vibrational modes of the phosphate groups could be established unequivocally due to the mass effect as a result of O^{18}. Attempts were made to exchange 90% of the apatite O^{16} by treating the samples with excess H_2O^{18} at higher temperatures and pressures. The choice of variables, temperature, pressure and time to effect this exchange was dictated and limited by the physical properties and chemical composition (SiO_2) of the vycor tubing used for the reaction chamber. The maximum O^{18} exchange obtained thus far, about 50%, was effected by treating a 3 to 7 molar mixture of $Ca(PO_3)_2$ and $CaCO_3$ at 20 atmospheres H_2O^{18} pressure at 900° for two hours. The total O^{16} to O^{18} proportion was 1 to 5. Treatment at higher temperature, pressure and longer time did not increase the O^{18} exchange; lack of increased exchange under these conditions may be due to O^{18} exchange of the H_2O^{18} with O^{16} of the vycor (SiO_2) tubing. An inert tube liner will be used in effort to increase the O^{18} exchange.

The infrared spectra of the sample containing about one-half of the total oxygen as O^{18} showed distinct band shifts which confirm that the previous assignments for the ν_3, ν_1, and ν_4 phosphate modes are correct. However, the weak HA band at 472 cm^{-1}, which has been assigned to a $\nu_3 - \nu_4$ difference tone by several investigators, shifts on O^{18} substitution by the calculated amount for the ν_2 phosphate vibration.

The band intensity, deuteration and preliminary O^{18} data indicate that the weak HA and HFA bands at 472 cm^{-1} are the ν_2 phosphate mode, and that the bands at 342 cm^{-1} in HA and at 355 to 345 cm^{-1} in HFA arise from translation motion of the OH groups.

The isostructural analogues of HA, SrA and BaA were prepared and examined by infrared spectroscopy to aid in the assignments of the HA low-frequency bands. It was necessary, therefore, to assign the SrA and BaA absorption bands. The major phosphate bands were assigned for both compounds and the OH modes for SrA. The OH modes of the BaA have not been unequivocally assigned due to the low intensity of the bands. The OH motions are of primary importance, particularly the OH librational motion which has been shown to be very sensitive to its molecular environment. The phosphate bands in SrA shift about 12 cm^{-1} relative to the corresponding phosphate bands in HA whereas the OH stretching band shifts 20 cm^{-1} and

the OH librational band shifts 93 cm^{-1}. The about eight-fold shift of the OH librational band shift relative to the phosphate band shift further illustrates the sensitivity of this mode to its molecular environment.

Significance to Dental Research:

Characterization and assignment of the infrared absorption bands of pure synthetic apatites and band changes caused by the presence of, for example, fluoride, chloride and carbonate, are essential in better defining the composition of the inorganic phase(s) of teeth and bones.

The amount of carbonate present in enamel has long been thought to have some bearing on relative susceptibility of teeth to caries. Carbonate has also been labelled by some investigators as essential for apatite formation. The exact location of carbonate within the crystal lattice and verification of the role of carbonate in apatite formation are essential to an understanding of its influence on caries susceptibility.

Some of the postulated reactions of fluoride with enamel, such as replacement of hydroxyl and carbonate groups, have not been clearly established by chemical experiments. Knowledge of the exact reaction(s) of fluoride, which accompany reduction in caries, would enable better selection of chemical conditions to maximize the reaction(s).

Proposed Course of Project:

Completion of O^{18} isotopic exchange experiments in order to assign with certainty the low-frequency apatite infrared absorption bands. Characterization of bands in this region will give additional information to aid in interpreting the structural features of apatites containing biologically important anions, such as, fluoride and carbonate.

Completion of the infrared studies of hydroxy-fluorapatite and carbonate containing apatite.

Part B: not included

250

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Odontopathic Bacterial Plaques: Etiologic Factors, Pathological Sequelae, Therapeutic Measures

Previous Serial Number: NIDR-28

Principal Investigator: Dr. P. H. Keyes

Other Investigators: Dr. R. J. Fitzgerald, Dr. H. V. Jordan, Dr. R. J. Gibbons, Dr. R. H. Larson, Dr. A. J. Gwinnett, Dr. P. N. Baer

Cooperating Units: None

Man Years:

Total:	4 1/4
Professional:	2 1/4
Other:	2

Objectives:

The objectives of the work described in this project still remains: (1) to attain a better understanding of dental plaque infections. Answers are needed to questions regarding the etiological factors involved, pathological features, and therapeutic measures of prevention and cure.

Methods Employed:

Animal model systems which use Syrian hamsters have been and continue to be of value. In vitro methods are also employed for assessing factors conducive to plaque formation and its control.

Experiments and Major Findings:

1. Studies in collaboration with Robert J. Fitzgerald demonstrated that dextranase products produced by colleagues at Merck and Company will prevent the accumulation of coronal plaque and smooth surface caries in animals subjected to appropriate dietobacterial challenges, i.e., sucrose and dextranogenic streptococci.

251

2. Studies in collaboration with Harold V. Jordan demonstrated that important differences in the disease response of hamsters followed the interactions between diets containing various amounts of sucrose and starch and plaque-forming bacteria, namely detranogenic streptococci and levanogenic diphtheroids. As the sucrose content of the diet decreased and the starch content increased, the population of streptococci fell and that of diphtheroids increased; caries activity and severe ulcerative gingival disease decreased; progressive periodontal pathosis became the only detectable disturbance.

Until recently it has not been possible to induce periodontal syndromes by inoculating human diphtheroids into the mouths of hamsters, although the isolates used appeared to be very similar to the Odontomyces viscosus, previously described as having the potential to form plaque and periodontal pathosis in this species. Therefore it has been encouraging to find that hamsters fed a diet containing starch have developed typical cervicoradicular plaque, gingival distortion, and alveoloclasia following the inoculation of human diphtheroids into their mouths.

3. Collaborative studies with Ronald J. Gibbons have demonstrated that in hamsters the addition of dextran to diets containing sucrose reduced plaque formation and caries activity.

4. Studies in collaboration with Rachel H. Larson revealed that rats designated by researchers at Michigan State University as caries-resistant were not resistant to either crevice decay, plaque formation, or to smooth surface decay when subjected to appropriate dietobacterial challenges.

5. Methyl $^{2-}$cyanoacrylate was mixed with several inorganic salts, some of which contained fluoride, and applied to extracted human teeth and to the teeth of Syrian hamsters. A. John Gwinnett examined sections of these human teeth and found satisfactory penetration of the sealant into the enamel surface. No decalcification of surfaces treated in this manner occurred while such teeth were incubated in culture medium containing caries conducive streptococci. The same findings were noted in the teeth of hamsters. However the material tended to soften and not to retain its bonding with the surface. Work with this compound stopped, after it was learned that this isomer of cyanoacrylate hydrolyses and is not biostable.

6. Studies in collaboration with Paul N. Baer showed that an infectious and transmissible flora was responsible for plaque and calculus formation in rats.

Significance to Dental Research:

1. Any substances which will assist in the dispersion of adherent pathogenic plaques on the surfaces of teeth will be of value in the prevention and cure of dental plaque infections. Clinical trials

with dextranase are now anticipated. It may be possible to supplement the antiplaque benefits of such enzymes with antibacterial agents, if the clinical trials prove beneficial.

2. The importance of dietary residues in the etiology of periodontal syndromes has not been generally recognized. The finding that starch enhances the periodontal syndrome in hamsters appears to be in coincidence with the observations that human populations which consume large quantities of cereal products may have serious root surface disease, i. e., radicular infections, and associated periodontal detachment. The successful implantation of human filament forming bacteria in mouths of hamsters and the subsequent development of periodontal pathosis may lead to animal model systems for assessing the pathogenic potential of human bacteria isolated from human patients with cervico-radicular infections.

3. The observation that additions of dextran to the diet can attenuate plaque forming reaction with sucrose suggests that food additives might be found which would be somewhat beneficial in man.

4. The use of sealing agents to seal vulnerable occlusal crevices in teeth (pits and fissures) could be of great value in preventing carious lesions in retention sites on teeth. Limited observation in vitro suggests that these products are highly effective, and clinical observations elsewhere are in agreement. However, real progress in this area will have to await the development of sealing agents which are bio-staple and thus adhere to enamel surfaces for long periods of time.

Proposed Course of Project:

1. Dextranase will be assessed in clinical trials. Human volunteers who harbour dextran producing streptococci will dissolve cubes of sucrose periodically during the day for several days. Dextranase will be administered in various formulations during or after the experimental period to determine its potential either to prevent or to displace plaque accumulations.

2. Tests in hamsters will be used to gather further insight into dieto-bacterial factors associated with periodontal pathosis. If bacterial isolates from humans can be shown to colonize on the cervico radicular surfaces of teeth and thereby induce gingivitis and periodontal syndromes, significant progress may be made in our understanding of "periodontal disease". Efforts are now in progress to let a contract with the Lincoln State School, Lincoln, Illinois, whose staff members are interested in defining the plaque infection status of mentally retarded persons under their care. Cultures from the mouths of these patients will be assessed in hamsters and in vitro.

3. A new sealing material has been prepared by Ethicon Inc. (sole distributors of Eastman Kodak's cyanoacrylates) for experimental trials in animals and in vitro. Isobutyl 2-cyanoacrylate and a special filler will be applied to extracted human teeth, which will then be sectioned and examined histologically (in collaboration with A. John Gwinnett, Dalhousie University, Halifax). These specimens will also be incubated with streptococcal cultures to determine whether the material will prevent decalcification of the coated surface. It will also be applied to the teeth of hamsters subjected to caries conducive conditions.

4. In vitro methods are being developed which may permit meaningful assays of the ability of antibacterial formulations to retard plaque formation and the potential of fluoride preparations to attenuate demineralization of enamel surfaces.

Part B

Publications:

1. Englander, H. R., Keyes, P. H., and Gestwicki, M. Clinical anticaries effect of repeated topical sodium fluoride applications by mouthpieces. J. Am. Dent. Assoc. 75:638-644, 1967.

2. Prevention and conservation in dentistry: a symposium. Bull. Acad. Med. N. J. 13:160-166, 1967.

3. Keyes, P. H. Odontopathic infections in the golden hamster: its biology and use in medical research. Eds. R. A. Hoffman, P. F. Robinson, and H. Magalhaes. Iowa State Press. Ames. 1968.

4. McCabe, R. M., Keyes, P. H., and Howell, A., Jr. An in vitro method for assessing the plaque forming ability of oral bacteria. Arch. Oral Biol. 12:1653-1656, 1967.

5. Fitzgerald, R. J., Keyes, P. H., Stoudt, T. H., Spinnell, D. M. The effects of a dextranase preparation on plaque and caries in hamsters. A preliminary report. J. Am. Dent. Assoc. 76:301-304, 1968.

6. Keyes, P. H. A review of dental caries. J. Am. Dent. Assoc. 76: June 1968.

7. Gwinnett, A. J. and Matsui, A. A study of enamel adhesives. The physical relationship between enamel and adhesive. Archs. Oral Biol. 12:1615-1620, 1967.

8. Gwinnett, A. J. Scandium as a target material for microradiography. J. Dent. Research. 46:1479, 1967.

254

Serial No. NIDR-54 (67)
1. Histology and Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Fate of Microorganisms Inserted into Healthy Gingival Pockets.

Previous Serial Number: None

Principal Investigator: Dr. Jens Waerhaug

Other Investigators: Dr. Harold Jordan and Dr. Paul Keyes

Cooperating Units: Dr. W. Titus, Laboratory Aids Branch, Division of Research Services

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

To study the fate of bacteria inserted into the potential space between the tooth and its normal epithelial cuff.

It has become increasingly clear that periodontitis is a bacterial disease and that the point of attack of the bacteria is at the gingival margin or in the gingival pocket. According to criteria outlined by one of the investigators (Dr. Jens Waerhaug) a healthy gingival pocket is one in which the normal epithelial cuff (attachment) adheres to the tooth in its full width without interjacent plaque. There are extreme differences of opinion, however, concerning the presence or absence of bacteria in this potential pocket. One group maintains that the healthy gingival pocket is essentially sterile and that bacteria which are introduced mechanically are eliminated. The other group holds that all such pockets always harbor bacteria.

Methods Employed and Major Findings:

The dog was chosen as the experimental animal partly because the anatomy of the gingival area is very similar to that of humans, and partly because the dog is handled rather easily without anesthesia.

In order to differentiate experimentally introduced microorganisms from those normally present on the teeth and their gingivae it is necessary to implant microorganisms which have been made resistant to a certain antibiotic. By culturing subsequent samples in media which contain sufficiently high concentrations of the antibiotic, growth of all but the resistant microorganisms is prevented.

It was hoped originally that one of the established hamster or rat strains of plaque forming bacteria could be used for experimental implantation in the dogs. As a consequence, in the first experiments the capacity of one such strain (T6 1600) which had been made resistant to streptomycin, to colonize in the experimental animal was investigated. The tongue and gingival areas of one dog was swabbed with 48 hr. cultures of T6 1600 for 10 min. and in addition 5 ml of the broth was added to the drinking water. Samples taken from the dog 1 and 4 days after inoculation showed no growth.

Since sucrose favors growth of T6 1600 in rodents, a soft diet was prepared by mixing equal amounts of laboratory chow pellets and confectioners sugar with a suitable amount of water. Two dogs were fed this diet for 10 days before inoculation with T6 1600 according to the technique previously described. The inoculation was repeated 2 days later. Again samples taken after 1 and 4 days showed no growth on the streptomycin containing medium.

The inability of the T6 1600 to colonize in the oral cavity of the dog made it useless for the present purpose and it was necessary to find a microorganism which was indigenous to the dog. Samples were collected from a fairly old dog with ample amounts of supra-and small amounts of subgingival plaque. A number of filament-forming bacteria and diphtheroids were isolated from the dog samples. However, it has been very difficult to subculture these initial isolates, a necessary step in the development of streptomycin resistant strains. pH determinations suggest that the saliva of the dog has a much higher pH than that of the rodents, a factor which may explain some of the difficulties.

Significance to Dental Research:

The establishment of whether or not known plaque-forming microorganisms are eliminated when introduced mechanically into the normal epithelial cuff is of the utmost importance for a correct appreciation of the pathogenesis of gingivitis and periodontal destruction.

Proposed Course of Project:

The investigator's tenure of appointment ceases in August 1968. A continuation of the program past this date is not planned.

Part B not included

257

Report of the Biometry and Field Investigations Branch
National Institute of Dental Research
Summary Statement

In the past year, the existing Epidemiology and Biometry Branch was reorganized into the Biometry and Field Investigations Branch. This reorganization included the establishment of three sections: Biometry, Clinical Trials, and Epidemiology. Drs. James P. Carlos, Harold R. Englander, and Norman W. Littleton, respectively, were appointed chiefs of these newly established sections. This reorganization provides the basis for implementing a broad, well-balanced program of field studies of oral diseases. In addition, biometric capabilities have been strengthened. Emphasis is now being given to the development of a computer-oriented data processing system. This system also will be used to investigate the methodology of design, estimation and analytical techniques in clinical trials.

During the year, research activities of the branch included field studies of dental caries, periodontal disease, and malocclusion. Some of these studies were initiated during the current year; others represent a continuation and expansion of productive efforts which began the previous year. In addition to direct research activities, members of the branch participated in collateral projects which included consultations on the design and conduct of field studies being undertaken by others and biometric services to the professional staff of the Institute.

Dental Caries

Dental caries activity in hamsters can be prevented completely by a fluoride containing gel topically applied to the teeth by use of a fitted vinyl mouthpiece. A field study was undertaken to test the effects of a similar regimen on dental caries activity in children living in an area with a fluoride deficient water supply. Water soluble gels containing either 1.1 percent sodium fluoride and 0.1 M phosphate (pH 4.5), or 1.1 percent sodium fluoride (pH 7.0) were used. These gels were topically applied to the teeth of children for six minutes per day by use of fitted vinyl mouth applicators.

After 21 months, comparisons with an untreated control group indicated that dental caries increments were importantly lower in children receiving either type of gel. Differences in the increments of DMF teeth averaged about 65 percent lower while differences in DMF tooth surfaces averaged 75 to 80 percent lower in treated children. Eleven months after fluoride gel applications had been stopped, dental caries increments were still importantly lower for treated children than for those in the control group. But differences in dental caries increments at this examination were not as great as those observed during the 21-month period of fluoride application. This may indicate that there is a falling off of protection after repeated topical fluoride therapy is discontinued. Children in this study will be examined again to determine if this trend continues.

Analyses of exfoliated deciduous teeth showed that the outer layers of enamel of teeth from treated children acquired significantly more fluoride than was found in the enamel of teeth from untreated children. Six to 9 months after fluoride applications were stopped, the outer layers of enamel still contained high concentrations of fluoride (2800 ppm).

This project has demonstrated the value of a dental caries preventive technique that can be used in conjunction with other appropriate caries inhibitory measures. It has also raised the question of the minimum concentration of fluoride in enamel necessary for an optimum anti-caries effect.

Another study of the anti-caries effects of repeated topical fluoride gel applications (3 times per week) was initiated in a fluoridated community in November 1966. This project will demonstrate whether additional caries inhibition can be obtained from repeated topical fluoride therapy in children using fluoridated water continuously from birth. Initial dental examinations for this study were completed in November-December 1966. Children in the experimental and control groups had 2.7 and 2.6 DMF teeth, respectively.

Deciduous teeth treated with sodium fluoride gel prior to exfoliation progressively acquired more fluoride as the number of treatments increased. Most of the acquired fluoride was restricted to the outer 20 microns of enamel. For example, teeth receiving an average of 56 topical sodium fluoride treatments had 1,785 ppm in the outermost enamel layer as compared to 922 ppm fluoride for untreated control teeth from children consuming only fluoridated water. Follow-up examinations of children in this study are scheduled in May 1968.

A third study of daily topical fluoride gel applications has been initiated in preschool children who are dependents of U.S. Coast Guard personnel. The primary purpose of this study is to determine if such treatment can maintain the deciduous dentition of these children free from the development of smooth surface lesions. Children participating in this study were first examined in November 1967, and are to be reexamined in June 1968.

Oral microorganisms (dental plaque) and intra-oral, food residues have been implicated in the etiology of dental caries. Studies of the interaction of these factors in persons nourished by stomach tube were continued during the year. Plaque material from tube-fed persons was found to be much less acidic when suspended in 10 percent solutions of sucrose, glucose, fructose, invert sugar or starch than plaque material from persons fed by mouth. Bacteriological study showed that total streptococcus counts were lower in plaque from tube-fed persons. Filamentous bacteria, lactobacillus and streptococcus salivarius types were isolated less frequently from either plaque or from swabs of the oral mucosa obtained from tube-fed persons. Comparisons between groups clearly indicated that the acidogenic properties and certain bacterial components of plaque obtained from tube-fed persons differed from plaque obtained from persons who were fed by mouth. However, within group comparisons failed to establish any consistent relationship between these variables.

Another study was undertaken in this population to determine the effects of sucrose, invert sugar and starch on the acidogenic properties and certain bacterial components of dental plaque when these carbohydrates were given orally to persons who had been nourished previously via stomach tube.

A substantial increase in the acidic potential of plaque material from tube fed persons was observed 30 days following oral administration of these carbohydrates. Exposure to sucrose or invert sugar, however, seemed to enhance the acidic properties of plaque to a greater extent than starch. Thirty days after the cessation of oral carbohydrate supplementation, the acidogenic capacity of plaque taken from these persons reverted to pre-test levels. Oral administration of these carbohydrates, however, had little effect on total counts of either streptococci or lactobacilli in plaque material obtained from these tube-fed persons.

The availability for study of persons nourished solely by stomach tube provides a unique opportunity to investigate the dynamic interactions between oral microorganisms and intra-oral food residues in the etiology of oral diseases. Plans have been made to expand these initial studies of the effects of selected carbohydrates on the oral microflora when these foods are introduced orally under well controlled, experimental conditions to persons nourished by stomach tube.

Certain strains of streptococci are known to cause dental caries in experimental animals. Strains have been isolated from humans which are cariogenic when implanted under proper conditions within the mouths of either rats or hamsters. Distinctive cultural characteristics of these types of streptococci have been identified. As a result, it seems expeditious to undertake epidemiological investigations of the occurrence of these microorganisms in human populations, and to determine if the observed distributions relate to dental caries activity.

A study of the distribution of streptococci which have cultural characteristics of microorganisms that are caries-conducive in animals is included in the fluoride-gel studies. Such streptococci were found to be widely distributed in these populations; they were recovered in high numbers from dental plaque; and their presence was correlated with the past dental caries experience.

Caries-conducive streptococci were not recovered from 96 percent of the caries-free two-year old children included in one of these studies.

Plans are being made to extend investigations of the occurrence of these "caries-conducive" streptococci to other populations.

Periodontal Disease

Field studies conducted by this branch show that a spectrum of clinical signs indicative of periodontal disease are intimately associated with intra-oral irritants such as plaque and calculus deposits. But the role of these and other local irritants in the etiology and pathogenesis of periodontal disease, a chronic and progressive process, is not adequately understood.

A long-term investigation of periodontal disease has been undertaken in collaboration with the Oral Medicine and Surgery Branch to study the influence of various intra-oral irritants on the initiation and progression of periodontal disease in a stable, adult male population. Base line data for this study have been collected from about 600 adult males living in Metropolitan Washington, D.C. Computer programs for the analysis of these data are being developed. A second series of examinations in this population is planned for September 1968.

The branch also is participating in a study of the effects of periodic dental prophylaxis and instruction in oral hygiene in the initiation and progression of periodontal disease. This program is being conducted in collaboration with the Dental College and Hospital, Lucknow, India, and is supported by PL 480 funds. Initial examinations have been obtained from about 2500 persons, and appropriate treatment regimens are in progress. It is estimated that the first meaningful findings will be available in 1969.

Occlusion

Recent investigations indicate that a derangement in vitamin D_2 metabolism during pregnancy may be responsible for the cardiovascular anomalies of the supravalvular aortic stenosis syndrome (SASS), especially when associated with idiopathic infantile hypercalcemia. Peculiar "elfin" facies and dental anomalies, especially malocclusion, are reported to be common features of SASS. A study was undertaken to determine if SASS, including the characteristic facial and dental features, could be produced in offspring by exposing pregnant rabbits to large doses of vitamin D_2.

Through a series of test dosages it was determined that about 750,000 units of the vitamin is sufficient to produce most of the anomalies characteristic of SASS. Various developmental defects of the cranio-facial complex were observed in the offspring of rabbits given large doses of vitamin D_2. Underdevelopment of the mandible resulted in dysgnathia and dental cross-bite. The abnormal osseous development responsible for the malocclusion occurred in 65 percent of these offspring. Differences were noted in the timing of suture and fontanel closure. Many of the bones of the cranio-facial complex were smaller, malformed, and had altered contour. Enamel hypoplasia of the central incisors was noted in 95% of the test offspring. None of these dental and facial abnormalities were noted in the offspring of rabbits in the control groups.

Experimental findings in the vitamin D_2 treated rabbits resemble anatomically the skeletal and orthodontic manifestations of SASS that have been reported in humans. Further use of this experimental technique may lead to a better understanding of the effects of hypervitaminosis D on growth and development.

Orthodontic care claims a considerable share of funds spent to improve dental health, but the natural history of occlusion has received little emphasis in epidemiological study. A serious deterrent to progress in the epidemiologic study of malocclusion has been the lack of a comprehensive, valid and reliable index of occlusal disorders for use in field investigations of large populations.

Recently a new index was proposed which is based upon sound statistical and orthodontic principles and may be useful for the study of the distribution of occlusal deviations from an ideal "standard." In collaboration with dental personnel from the Division of Health Examination statistics, National Center for Health Statistics, an investigation has been initiated to determine the reproducibility of the TPI (Treatment Priority Index) and the level of agreement as to the severity of malocclusion attained when index distributions are compared with the clinical judgment of orthodontists.

A long-term study of occlusion also is in progress and it involves the reexamination of children at ages of critical tooth emergence and jaw development. To date, the results of this study indicate that a parabola computed from direct measurements of arch length and breadth adequately describes the size and shape of the dental arches. Malalignment of the dentition seems to be inversely related to arch breadth, but unrelated to either tooth size or arch length. Relationships established during the course of this study may prove valuable in understanding the sequence of events that lead to the various occlusal forms.

Clefts of the lip and/or palate frequently show familial predisposition; the majority of cases, however, do not show clear-cut evidence of a simple genetic mode of transmission. It has been reported that certain oro-facial aberrations occur with greater frequency and consistency in the near kindred of cleft propositi. It has been suggested that such aberrations may be minor expressions of congenital oro-facial clefts. In cooperation with Human Genetics Branch, a study was done to determine if selected dental and oral morphological aberrations occurred with greater frequency in the near kindred of cl/cp individuals when compared with a control population. Examinations were conducted on a total of 93 families with one or more cl/cp propositi. Results were compared with those obtained from 82 control (non-cleft) families. With the exception of a greater number of minor palatal defects in the index families, no significant differences were observed between these populations. At the present time, the usefulness of including these defects in genetic and epidemiological studies of cl/cp seems quite limited.

Biometric and Collateral Activities

Considerable effort has been given by personnel of the Biometry Section to the design and development of a computer-oriented data system to produce faster and more detailed analyses of data gathered in the clinical testing of potential caries preventatives, and in epidemiologic surveys. This system is based upon the field collection of data in a form suitable for automatic production of punched cards by an Optical Mark Page Reader. Data reduction and analysis is accomplished on the NIH IBM 360/50 computer using algorithmic language programs developed by Section personnel. The system is expected to be fully operative by July, 1968 and will be available for use by investigators conducting such studies.

The Biometry Section has also begun a broad program of investigation into the methodology of optimum design, estimation and analytical techniques in dental trials.

263

In addition to direct research activities and biometric services to our professional staff, a considerable amount of time was devoted to consultation in the design, conduct, data processing, and analyses of the results of field studies undertaken by others.

During the year, plans have been made to offer residency programs in Biometry and Field Investigations to help meet the need for additional trained personnel in this area. Application has been made to the American Dental Association for designation of this program as an approved residency. It is anticipated that the first residents will be appointed during the coming year.

Serial No. NIDR-55 (68)
1. Biometry and Field Investigations Br.
2. Biometry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A:

Project Title: Studies on the Design and Analysis of Dental Clinical Trials

Previous Serial Number: None

Principal Investigator: Dr. James P. Carlos

Other Investigator: Mr. Rickley S. Senning

Cooperating Units: None

Man Years

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

1. To develop a rationale for optimum allocation of subjects in dental caries clinical trials by studying the quantitative effects of various predictor variables on the incidence of caries.

2. To investigate unbiased methods of estimating the true increment of new carious lesions, when the clinical observations are subject to error.

3. To study the power and efficiency of various statistical tests of hypotheses in analyzing data from caries clinical trials.

Methods Employed:

Several mathematical models of dental caries clinical trials have been developed and are under study to determine their predictive validity in terms of data actually observed. With these models, the effect of misclassification error in the data can be simulated, and its consequences in tests of hypotheses observed, when various procedures are used to estimate the true incidence of caries during a study.

Part A (continued)

Standard multivariate statistics and simulation techniques are used to evaluate the relative efficiency of matched, balanced and random designs in dental clinical trials and the appropriateness of alternative significance tests of the data.

Major Findings:

Using one of the models referred to above, it has been demonstrated that estimation procedures conventionally used in dental trials are biased and usually result in overly conservative tests of significance with increased probability of failure to recognize an effective agent.

Procedures for computing unbiased estimates of the parameters of this model have been developed.

Preliminary data suggest that matched and balanced designs commonly used in dental trials have little theoretical foundation and probably result in reduced experimental efficiency.

Proposed Course of Project:

The project is continuing.

Part B:

Publications:

Carlos, J. P. and Senning, R. S. Error and bias in dental clinical trials. J. Dent. Res. 47:142-148; Jan-Feb, 1968.

Serial No. NIDR-56 (64)
1. Biometry and Field Investigations Br.
2. Clinical Trials Sec.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A:

Project Title: Clinical Anti-Caries Effect of Repeated Topical Sodium Fluoride Applications by Mouthpieces

Previous Serial Number: NIDR-45

Principal Investigator: Dr. H. R. Englander

Other Investigators: Drs. James P. Carlos and Harold V. Jordan, Jr.

Cooperating Units: Cheektowaga Central School District No. 1; James R. Mellberg, Research Division of the Kendall Company, Barrington, Illinois

Man Years:

Total:	1-1/4
Professional:	3/4
Other:	1/2

Objectives:

The first two years of this project were supported by an NIDR Contract No. PH 43-64-944. The original purpose of this project was to test the anti-caries effect of water soluble gels containing 1.1 per cent sodium fluoride when the gels were applied daily for six minutes in mouth applicators to the teeth of children living in fluoride-deficient Cheektowaga, New York.

The acquisition of permanently bound fluoride by enamel of deciduous teeth from the topical applications, and the occurrence and distribution of caries-conducive streptococcal strains has also been studied.

The children in the study have not applied sodium fluoride/in mouth applicators during the last two years. Therefore, additional data have been collected to determine the residual anti-caries effect of the fluoride applications after they had been discontinued.

Part A (Continued)

Furthermore, the data from the entire series of examinations are being analyzed to observe the effect on caries experience in clinical trials of the level of community dental care, the designation of questionable carious lesions in the examination technique, and the possible influence of positional errors on the interpretation of relative caries increments. In the future the data will be analyzed so that it will be possible to separate smooth surface from pit and fissure caries increments.

Methods Employed:

Clinical examination of approximately 500 children, initially aged 11-14 years, who were randomly assigned to groups receiving the sodium fluoride gels and a control group not receiving the gels. Statistical evaluation of data collected during the 21-month period in which the gels were applied, and of data collected 11 and 23 months after the gels had been discontinued. Chemical analysis of the fluoride concentration in the outer enamel layers of exfoliated deciduous and extracted permanent teeth, and bacterial culture of dental plaque.

Major Findings:

After 21 months, children applying an average of 245 repeated fluoride gel applications developed about four-fifths fewer DMFS than children in the control group not applying the fluoride gels.

Eleven months after the repeated topical fluoride gel applications had been discontinued, dental caries increments were importantly lower in the children that had applied the fluoride gels than in the controls. However, the differences in increments between the fluoride gel and control groups were not as great when compared with the differences obtained during the 21-month period of the repeated applications.

During the 21-month period of fluoride gel applications, the amount of permanently bound fluoride acquired by deciduous enamel increased with the number of repeated fluoride applications received before exfoliation.

The outermost layers of enamel of deciduous teeth exfoliated 6-9 months after the fluoride applications had been stopped contained high concentrations of fluoride (about 2800 ppm). This indicated that very little fluoride had been lost from enamel during the period that fluoride was not applied.

No differences could be found in the ability to recover caries-conducive streptococci from the dental plaques of children applying and not applying the fluoride gels.

Part A (Continued)

Significance to Dental Research:

This project has demonstrated the striking anti-caries benefits that can be achieved with repeated topical fluoride treatments by mouth applicators. Furthermore, it has demonstrated the importance of considering the fluoride uptake of teeth in the evaluation of clinical trials with fluorides.

The data on dental caries increments after the fluoride applications had been discontinued do not indicate that there is a falling off of protection during this period, and may lead to the recommendation that repeated topical fluoride applications can be discontinued in the treatment of rampant caries cases in order to obtain optimum anti-caries benefits when the enamel has acquired 2000-3000 ppm fluoride from topical applications.

Proposed Course of Project:

The children will be examined in May 1968 to evaluate the residual anticaries effect 23 months after the discontinuance of the fluoride applications.

Part B

Publications:

Englander, H. R., P. H. Keyes and M. Gestwicki: Clinical anti-caries effect of repeated topical sodium fluoride applications by mouth pieces. JADA 75: 638, Sept. 1967.

Mellberg, J. R., H. R. Englander, and C. R. Nicholson: Acquisition of fluoride in vivo by deciduous enamel from daily topical fluoride applications. J. Oral Ther. & Pharm. 3: 330, March 1967.

Mellberg, J R., H. R. Englander, and C. R. Nicholson: Acquisition of fluoride in vivo by deciduous enamel from topical fluoride applications over 21 months. Arch. Oral Biol. 12: 1139, Oct. 1967

Englander, H. R.: Views on the rationale of topical fluoride therapy. JACD 35: 15, Jan. 1968.

Jordan, H. V., H. R. Englander and S. Lim: The presence of potentially cariogenic streptococci in various population groups. Preprinted Abstracts (370) IADR 46th Gen. Meeting, March, 1968.

Serial No. NIDR-57 (66)
1. Biometry and Field Investigations Br.
2. Clinical Trials Sec.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Anti-Caries Effect of Repeated Topical Fluoride Applications in a Fluoridated Community

Previous Serial Number: NIDR-46

Principal Investigator: Dr. Harold R. Englander

Other Investigators: Dr. Harold V. Jordan and Dr. James P. Carlos

Cooperating Units: Central Piedmont Community College, Charlotte, North Carolina, Drs. Barry G. Miller and Harry Snyder; Mecklenburg County Health Department, Charlotte, North Carolina, Dr. Luby Sherrill; The Research Division of the Kendall Company, Barrington, Illinois, Mr. James R. Mellberg.

Man Years

Total:	1-1/4
Professional:	3/4
Other:	1/2

Objectives:

This study is being supported by contract number PH 43-67-60. The principal purpose of the project is to determine whether frequently repeated topical treatments with a concentrated sodium fluoride gel in mouth applicators can further reduce the low dental caries activity in children consuming fluoridated water continuously from birth. Such applications will be made three times weekly for three minutes over a period of three years.

Exfoliated deciduous teeth are also being collected from the children in order to determine how much additional fluoride can be acquired by enamel from the topical treatments over that acquired from consuming fluoridated water alone.

Part A (Continued)

The occurrence and distribution of caries-conducive streptococcal strains in the dental plaque of children in Charlotte is being determined as part of an epidemiologic survey. The effect of the fluoride gel applications on the prevalence of this strain in the plaque will be studied.

The usefulness of radiographs in addition to clinical examinations in the conduct of clinical trials will be evaluated.

Methods Employed:

Clinical and radiographic examinations of approximately 900 children, aged 11-14 years, randomly assigned to one group applying a gel containing 1.1 per cent sodium fluoride or to another group (control) not applying the gels. Statistical analysis and interpretation of data collected initially and at periodic intervals. Fluoride analysis of the outer enamel layers of exfoliated deciduous teeth, storing them in Jordan's transport medium, and plating and identifying the streptococci on mitis-salivarius agar.

Major Findings:

Initial dental examinations were completed in November-December 1966. Children in the experimental and control groups had 2.7 and 2.6 DMF teeth, respectively, and the status of gingival health and oral hygiene was similar for both groups.

The teeth treated with the sodium fluoride gel progressively acquired more fluoride as the number of treatments increased. Most of the acquired fluoride was restricted to the outer 20 microns of enamel. For example, teeth receiving an average of 56 topical sodium fluoride treatments had 1,785 ppm fluoride in the outermost enamel layer as compared to 922 ppm fluoride for untreated control teeth from children consuming only fluoridated water.

About 70 percent of the children in this fluoridated community did not harbor caries-conducive streptococcal strains on their teeth. The repeated fluoride applications did not alter the occurrence and distribution of these microorganisms.

Significance to Dental Research:

The project will demonstrate whether an additional anti-caries benefit can be obtained from repeated topical fluoride therapy in children consuming fluoridated water continuously from birth. If so, this technique could be recommended in fluoridated areas for the management of cases of rampant dental caries.

Part A (Continued)

The fluoride analysis of teeth and the microbiological phases of this study will provide valuable information on the mechanism of fluoride action and the epidemiology of the caries-conducive streptococci.

Proposed Course of Project:

The thrice weekly gel applications will continue in school until June 1969. The follow-up clinical and radiographic examinations to determine the effect of the repeated fluoride applicators after 18 months will be conducted in May 1968.

Deciduous teeth will continue to be collected and analyzed for fluoride.

Urine specimens will be analyzed for fluoride to monitor possible fluoride ingestion.

Further plaque samples will be collected for culturing in May 1968.

Part B

Publications

1. Mellberg, J. R., Nicholson, C. R., Miller, B. G., and Englander, H. R.: Acquisition of fluoride by enamel from repeated topical sodium fluoride applications in a fluoridated area. J. Dent. Res. (In press).

2. Jordan, H. W., Englander, H. R., and Lim, S.: The presence of potentially cariogenic streptococci in various population groups. IADR Preprinted Abstracts (370) 46th Gen. Meeting, March 1968.

Serial No. NIDR-58 (68)
1. Biometry and Field Investigations Br.
2. Clinical Trials Sec.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Anti-Caries Effect of Repeated Topical Fluoride Treatments on the Deciduous Dentition

Previous Serial Number: None

Principal Investigator: Dr. Harold R. Englander

Other Investigators: Dr. Harold V. Jordan, Jr., Dr. James P. Carlos, and Dr. Peter J. Coccaro

Cooperating Units: United States Coast Guard, Governors Island, New York, New York, Captain William O. Engler, and Commander Edward D. Woolridge

Man Years

Total:	1-1/4
Professional:	3/4
Other:	1/2

Objectives:

The principal purpose of this project is to determine whether frequently repeated daily topical applications of a concentrated sodium fluoride gel can maintain the initially caries-free deciduous dentition of pre-school children free from the initiation of smooth surface dental caries. The population selected for this study are children, aged 2-7 years, who are dependents of U.S. Coast Guard personnel.

Exfoliated deciduous teeth are also being collected from the children applying the fluoride and placebo gels in mouthpieces in order to estimate how much fluoride is acquired by enamel from the topical treatments, and to estimate the fluoride levels in the surface enamel of teeth of children who remain caries-free in the fluoride group and in the enamel of those who develop caries in the placebo treated group.

Part A (Continued)

The distribution of cariogenic streptococci in the dental plaque of caries free 2 and 3 year-olds is being followed to study the relationship between the occurrence of these bacteria and the development of pit and fissure and smooth surface caries.

Methods Employed:

Clinical and radiographic examinations of approximately 400 children,aged 2-7 years, randomly assigned to one group applying a gel containing 1.1 per cent sodium fluoride and 0.1 molar sodium phosphate (pH 4.5) and to another group applying a neutral placebo gel. Statistical evaluation of data collected initially and at six-month intervals. Chemical analysis of the fluoride concentration in enamel from exfoliated teeth. Collecting plaque samples from each child and culturing for streptococci, lactobacilli, and other microbiota. Measurement of dental casts of the children for growth and development studies.

Major Findings:

The children were examined for dental caries, gingival health (PI) and oral cleanliness (OHI) in November 1967. Plaque was also obtained from a sample of the children.

Children in the gel and control group had 4.7 and 4.8 def teeth, respectively, and about 85 children in each group were caries-free. Group PI and OHI scores were similar. Ninety-six percent of the caries-free 2-year olds did not harbor cariogenic streptococci.

Significance to Dental Research:

The use of the deciduous rather than the permanent teeth for evaluating the potential of an anti-caries technique has seldom been used in field testing, but it has certain advantages. Inasmuch as the pits and fissures of deciduous molars are usually not as deep or as defective as those found in the permanent molars and proximal lesions are more readily detectable clinically, examiner error and bias is greatly reduced. Furthermore, since the carious lesions affecting deciduous molars initiate primarily on smooth surfaces, the value of the topical treatments in preventing smooth surface caries can be evaluated with greater facility.

A comparison of the fluoride levels in the teeth of children remaining caries-free with those control children developing extensive caries will provide information concerning the optimum fluoride concentration necessary for a maximum anti-caries effect.

Part A (Continued)

Studies on the plaque microflora will provide insight into the relations between the cariogenic streptococci and smooth surface, and pit and fissure decay.

Proposed Course of Project:

The repeated topical fluoride applications will continue for 2.5 more years. The next clinical examination will be conducted in May 1968. Deciduous teeth from each child will be collected for fluoride analysis. Further plaque samples will be collected for culturing.

Part B Not included.

Serial No. NIDR-59 (63)
1. Biometry and Field Investigations Br.
2. Clinical Trials Sec.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Experimental Dental Caries in the Syrian Hamster

Previous Serial Number: NIDR-48

Principal Investigator: Dr. Harold R. Englander

Other Investigators: Dr. Paul H. Keyes

Cooperating Units: None

Man Years

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives:

The anti-caries effect of dietary supplements with organic and inorganic phosphates has been evaluated. In other experiments the anti-caries effect of prophylactic pastes containing various fluoride formulations have been assayed.

Methods Employed:

1. A caries-conducive ration has been supplemented with either monosodium phosphate, diammonium phosphate, sodium trimeta-phosphate, or sodium phytate.

2. Infected animals fed a diet containing a high concentration of sucrose were given prophylaxes with silex or with abrasive pastes containing stannous fluoride or sodium-silico-fluoride.

Major Findings:

1. Significantly less cavitation developed in hamsters fed diets supplemented with the phosphates at a concentration of 2 percent.

Dental caries scores were lowest for the animals given the trimetaphosphate. When the phosphate compound concentrations were reduced to 0.5 percent only the scores for the trimetaphosphate group were significantly less than the controls. No anti-caries effect was observed when dietary supplementation with the phosphate compounds (0.5 percent) was made thrice weekly.

2. Animals given one-minute prophylaxes with abrasive pastes containing stannous fluoride plus zircate or sodium fluoride at comparable fluoride concentrations developed less cavitation in the maxillary second molars than control animals receiving a prophylaxis with fluoride-free pumice. However the zircate paste did not have a greater anti-caries effect than the sodium fluoride paste.

Significance to Dental Research:

1. When phosphate compounds are administered intermittently in a caries-test ration, their anti-caries effect tends to disappear. It is therefore unlikely they would have any important potential as an anti-caries agent when consumed intermittently by human beings.

2. Since the zircate paste containing stannous fluoride did not have a greater anti-caries effect than a paste containing sodium fluoride and there have been reports of its toxicity, it is doubtful that it should be recommended for clinical practice.

Proposed Course of Project:

The anti-caries effect of polyphosphates will be studied.

Evaluation of prophylactic pastes containing other fluoride formulations will continue together with a fluoride analysis of hamster teeth receiving varying degrees of protection with fluoride.

Part B

Publications

Englander, H. R. and Keyes, P. H.: Acid production in the dental plaque of hamsters protected from dental caries with sodium fluoride. J. Oral Thera. and Pharm. 4: 382, March 1968.

Keyes, P. H., Rowberry, S. H., Englander, H. R., and Fitzgerald, R. J.: Bioassays of medicoments for the control of dentobacterial plaque, dental caries, and periodontal lesions in Syrian hamsters. J. Oral Thera. and Pharm. 3: 157, Nov. 1967.

Serial No. NIDR-60 (65)
1. Biometry and Field Investigations Br.
2. Epidemiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Studies of Oral Health in Persons Nourished by Stomach Tube

Previous Serial Number: NIDR-44

Principal Investigator: Dr. N. W. Littleton

Other Investigator: Mr. R. M. McCabe

Cooperating Units: Sunland Hospital at Orlando, Orlando, Florida

Man Years

Total:	1-3/4
Professional:	1-1/2
Other:	1/4

Project Description:

Objectives:

Oral microorganisms (dental plaque) and intra-oral, food residues have been implicated in the etiology of oral diseases. The objectives of this study are:

1. To compare oral health status in persons nourished solely by stomach tube for long periods with that of persons who were fed orally.

2. To compare plaque material from tube-fed persons with that obtained from persons fed orally in regard to:
   a. Acidogenic properties
   b. Selected bacterial components

3. To compare the recovery of lactobacilli and streptococcus salivarius types from swabs of the oral mucosa obtained from tube-fed persons and persons fed orally.

4. To investigate the effects of sucrose, invert sugar and starch on the acidogenic properties and selected bacterial components of dental plaque when these carbohydrates are given orally to persons nourished by stomach tube.

Part A (continued)

Methods Employed:

The oral tissues of about 400 mentally retarded, physically handicapped persons were examined clinically. This population included about 75 persons who had been nourished by stomach tube for periods ranging from a few days to more than five years. Plaque material and swabs of oral mucosa were obtained from a subsample of these tube-fed persons. The pH of plaque material was determined in vitro with and without the addition of a 10 percent solution of sucrose, glucose or fructose. Plaque material, after appropriate serial dilutions, and swabs of the oral mucosa were plated onto selective culture media. Following incubation under 95 percent nitrogen and 5 percent carbon dioxide, growth of lactobacilli, filamentous bacteria and streptococci was estimated. Results on samples obtained from tube-fed persons were compared with those from similar samples obtained from a subgroup of persons fed orally.

In addition, a single carbohydrate--sucrose, invert sugar, or starch--was given orally for 30 days to persons fed by stomach tube. Plaque samples were obtained from these persons before, during and after oral supplementation. The effects of the oral administration of these carbohydrates on the acidic potential and bacterial composition of plaque obtained from tube-fed persons was investigated.

Major Findings:

Evidence of dental caries activity in this population was limited almost entirely to missing teeth. Teeth with either open carious lesions or restorations were observed rarely. Gingival and periodontal disease was widespread and severe, but clinical evidence of disease was particularly overt in tube-fed persons. Plaque was present in the mouths of all persons in this study, but these deposits were less extensive in persons fed by stomach tube than in persons who were fed by mouth. Abundant deposits of calculus were observed about the teeth of persons in both groups.

The pH remained above 6.0 over a one-hour experimental period following suspension of plaque material from tube-fed persons in 10 percent solutions of sucrose, glucose, fructose, invert sugar, or starch. Under similar conditions the pH of plaque suspensions from persons fed by mouth decreased substantially and reached levels in the range of 4.3 to 4.8 within 5 to 10 minutes. Limited bacteriological study indicated that total streptococcus counts were lower in plaque from tube-fed persons. Filamentous bacteria, lactobacilli and streptococcus salivarius types were isolated frequently from either plaque or from swabs of the oral mucosa

Part A (continued)

obtained from tube-fed persons. Between group comparisons clearly indicated that the acidogenic properties and certain bacterial components of plaque obtained from tube-fed persons differed from plaque obtained from persons who were fed by mouth. However, within group comparisons failed to establish any consistent relationships between these variables.

A substantial increase in the acidogenic potential of plaque material was observed 30 days following oral administration of sucrose, invert sugar or starch to persons nourished by stomach tube. Exposure to sucrose and invert sugar, however, seemed to enhance the acidic properties of plaque to a greater extent than starch. Thirty days after the cessation of oral carbohydrate administration, the acidogenic capacity of plaque taken from these persons reverted to pre-test levels. Oral administration of these carbohydrates, however, had little effect on total counts of either streptococci or lactobacilli in plaque material from these tube-fed persons.

In collaboration with Dr. Harold Jordan plaque material from a small sample of these children was examined for the presence of extracellular polysaccharide producing streptococci. These microorganisms were recovered from children eating by mouth, but they were not recovered from any of the 12 plaque samples obtained from tube-fed persons.

Significance to Dental Research:

The availability for study of persons nourished solely by stomach tube provides a unique opportunity to investigate the dynamic interactions between oral microorganisms and intraoral food residues in the etiology of oral diseases.

Proposed Course of Project:

Plans have been made to extend the study of oral diseases in this population. These plans include further study of the effects of selected carbohydrates on the oral microflora when these foods are introduced orally under well controlled, experimental conditions to persons nourished by stomach tube.

Part B

Publications:

Littleton, N. W., McCabe, R. M. and Carter, C. H.: Studies of oral health in persons nourished by stomach tube. II. Acidogenic properties and selected bacterial components of plaque material. Arch. Oral Biol. (in press).

Serial No. NIDR-61 (68)
1. Biometry and Field Investigations Br.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Production of "Elfin" Facies and Abnormal Dentition by Vitamin D_2 during Pregnancy: Relationship to the Supravalvular Aortic Stenosis Syndrome

Previous Serial Number: None

Principal Investigator: Dr. Loren F. Mills

Other Investigator: None

Cooperating Unit: National Heart Institute, Cardiology Branch, Dr. William F. Friedman

Man Years

Total:	1-1/4
Professional:	1/2
Other:	3/4

Project Description:

Objectives:

Recent investigations have suggested that a derangement in vitamin D_2 metabolism during pregnancy may be responsible for the cardiovascular anomalies of the supravalvular aortic stenosis syndrome (SASS), especially when the latter are associated with idiopathic infantile hypercalcemia. Peculiar "elfin" facies and dental anomalies, especially malocclusion, are reported to be common features of SASS.

The present study was undertaken to further investigate the characteristic facial and dental features of SASS.

Methods Employed:

Through a series of test dosages of vitamin D_2 it was determined that approximately 750,000 units of the vitamin is sufficient to produce most of the anomalies characteristic to SASS. Histologic and macro and microscopic techniques are being used for observation of the various teratological effects. Blood and bone samples have been submitted for calcium and phosphorus determinations.

Part A (Continued)

Major Findings:

Various developmental defects of the cranio-facial complex were observed in vitamin D offspring when compared to controls. Underdevelopment of the mandible resulted in dysgnathia and dental cross-bite. The abnormal asseous development responsible for the malocclusion occurred in 65 percent of the offspring. Differences were noted in the timing of suture and fortanel closure. Many of the bones of the cranio-facial complex were smaller, malformed, and had altered contour. Enamel hypoplasia of the central incisors was noted in 95% of the test offspring. None of these dental and facial abnormalities were noted in control offspring.

Significance to Dental Research:

The experimental findings in rabbits resemble anatomically the skeletal and orthodontic manifestations of SASS that have been reported in humans. Further use of this experimental technique may lead to a better understanding of the effects of hypervitaminosis D on growth and development.

Proposed Course of Project:

Further investigations are to be undertaken to (1) determine minimal dosage necessary to produce SASS (2) determine if defects of the craniofacial complex can be produced by hypervitaminosis D without SASS.

Part B

Publications

Friedman, William and Mills, L. F.: The relationship between vitamin D and the cranio-facial and dental anomalies of the SASS. (Submitted to Pediatrics Feb., 1968).

Serial No. NIDR-62 (62)
1. Biometry and Field Investigations Br.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Epidemiological Studies of Malocclusion

Previous Serial Number: NIDR-49

Principal Investigator: Dr. Loren F. Mills

Other Investigators: None

Cooperating Units: Board of Education, Prince Georges County, Maryland; West Liberty State College, West Liberty, West Virginia; United States Naval Academy, Annapolis, Maryland; Cleft Palate Clinic, Lancaster, Pennsylvania

Man Years

Total:	1-1/4
Professional:	1/2
Other:	3/4

Project Description:

Objectives:

To derive a direct and reproducible method for determining the size and shape of dental arches;

To develop criteria for assessing the prevalence and severity of occlusal anomalies in populations;

To apply these criteria in attempts to elucidate possible relationships between the factors under study.

Methods Employed:

By direct observation each individual in this study is examined for the following variables: 1) alignment of teeth; 2) maxillo-mandibular relationship; 3) length and breadth of the dental arches; 4) size and morphology of teeth; and 5) height, weight and other anthropometric characteristics.

Part A (Continued)

These methods are being applied to populations to obtain both long-term and cross-sectional data. The initial step in this investigation of malocclusion was a pilot study conducted in about 500 Midshipmen examined at the United States Naval Academy. The study was extended this year to include examinations of about 1000 college students, aged 18 to 24 years, at West Liberty State College. In addition, yearly examinations are obtained from about 300 school children, aged 6 to 18 years.

Major Findings:

Data obtained during the course of this study has indicated that a parabola computed from direct measurements of arch length and breadth adequately describes the size and shape of the dental arches. Tabulations indicate that malalignment of the dentition was inversely related to arch breadth. Size of teeth and arch length were apparently unrelated to the occurrence of malalignment. Results also indicate that in these samples the space occupied by the teeth in the dental arches decreases with age. This is consistent with the generally accepted concepts of approximal wear, mesial drift of teeth and difference in size between deciduous molars and bicuspids.

Significance to Dental Research:

Criteria developed during the course of this study could prove valuable in the investigation of orthodontic problems in populations. These criteria could aid in detecting the need for early preventive orthodontic care. In population studies of malocclusion obtaining dental casts is time consuming and expensive and therefore prohibitive in studies of large groups. This technique may obviate the need for dental casts in population studies concerning: (1) the effects of race, nutrition, climate, geographic location, or other environmental factors on the size and shape of the jaws, (2) malocclusion studies which estimate the space available for teeth and the effects of faulty mouth habits on jaw size, and (3) predetermining the appropriate size of the maxilla before cleft palate repairs are made by the plastic surgeon.

Proposed Course of Project:

Continue to analyze these data, and to test these findings in longitudinal study to evaluate their predictive potential. The future course of this study will be developed as the results of the data are analyzed and interpreted.

Part B

Publications

Mills, L F., Niswander, J. D., Mazaheri, M., and Brunelle, J.A.: Minor oral and facial defects in relatives of oral cleft patients. (Accepted for publication), Angle Orthodontist, Dec. 2, 1967.

Serial No. NIDR-63 (66)
1. Biometry and Field Investigations Br.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Serial Extraction Study on Preadolescent Children Having Crowded Class I Occlusion

Previous Serial Number: NIDR-51

Principal Investigator: Dr. Loren F. Mills

Other Investigators: Dr. Richard Christiansen

Cooperating Units: Board of Education, Prince Georges County, Maryland

Man Years

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives:

To compare the changes which occur within the jaws of subjects who undergo serial extraction and subjects with a similar diagnosis who do not receive extractions. Also to compare the degree and direction of the development of the facial bones.

Methods Employed:

Final screening of approximately 56 subjects will be conducted at the National Institutes of Health. The initial records secured on the 56 project subjects ultimately selected will include the following: (a) medical and dental histories; (b) detailed mouth examinations; (c) five cephalometric radiographs; (d) radiographs of individual teeth; (e) orthodontic models; (f) facial and intraoral photographs; and (g) medical examination. The sample will be divided into two groups of 28 subjects, each comprised of 14 boys and 14 girls.

Part A (Continued)

Major Findings:

In May and October of 1967, approximately 1500 children, between the ages of 6-12 years, were examined in four schools in Prince Georges County. Approximately 42 of these children fit the criteria for the study.

Significance to Dental Research:

Serial extraction procedure is designed to anticipate and hopefully prevent the development of a fully matured deformity in the permanent dentition. The procedure of serial extraction is used by some to help guide the eruption of permanent teeth into more favorable positions in the arches. This study will try to document the concomitant changes that occur dentally and skeletally and their frequency of occurrence.

Proposed Course of Project:

The data gathered to date will be utilized for a future publication. Plans for the future course of this study await necessary personnel and equipment. Reevaluation of diagnostic criteria is in progress.

Part B not included.

Serial No. NIDR-64 (61)
1. Biometry and Field Investigations Br.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Clinical Trial of a Dentifrice Containing Phosphate and Fluoride

Previous Serial Number: NIDR-53

Principal Investigator: Dr. L. F. Mills

Other Investigators: Dr. F. J. McClure and Mr. C. L. White

Cooperating Units: Lancaster Cleft Palate Clinic and Milton J. Hershey School

Man Years

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives:

To determine if a dentifrice containing a soluble phosphate and sodium fluoride will reduce the incidence of dental caries.

Methods Employed:

A dentifrice containing 10 per cent soluble phosphate and 0.25 per cent sodium fluoride is being tested in a double blind study at an institution with 900 boys residing in approximately 50 cottages. The cottages were matched by caries prevalence and equal numbers were randomly assigned to study and control groups. The control dentifrice is similar but contains no soluble phosphate or fluoride. A base line of caries prevalence was established by a bite wing radiograph and visual dental examination. Caries incidence will be determined from periodic repetition of the bite wing and visual examination. Mean incidence of carious teeth and tooth surfaces are to be compared for study and control groups.

Part A (Continued)

Major Findings:

Preliminary tabulations and analyses of the data failed to demonstrate any dental caries inhibiting effects of a tooth-paste containing sodium fluoride and 10 per cent soluble phosphate. Dental caries increments over a period of 36 months were essentially the same in both test and control groups.

Significance to Dental Research:

Dietary control of caries is impractical for large groups. Water fluoridation is not readily available to those who are not served by a communal water supply and has been denied to many others by the action of antifluoridationists or by the inaction of local authorities. It would be desirable to have available other effective methods for the mass control of dental caries to supplement fluoridation. Because tooth-brushing is a widespread established habit available to all, therapeutic dentifrices offer promise as a means of inhibiting caries. There is abundant evidence from animal experimentation that a soluble phosphate in the diet is capable of inhibiting caries. Animal experiments further indicate that the cario-static action of a soluble phosphate may be topical and hence its incorporation in a dentifrice to assure appreciable contact with tooth surface. Also, it has been reported that a topical solution containing fluoride and phosphate is more cariostatic than fluoride alone.

Proposed Course of Project:

This project was terminated in September 1967. Final analysis of the data is in progress. Even though the results were nega-tive, many interesting aspects of the study, heretofore unpublished, have merit and will be reported.

Part B not included.

Report of the Clinical Director
National Institute of Dental Research
Summary Statement

In our struggle to preserve and maintain the maximum degree of health and function of the oral cavity, certain trends in our clinical dental research program are becoming apparent.

The ideal of a dental researcher is to reach eventually a plateau of knowledge, understanding and control of dental problems that first the oral surgeon, then the endodontist, and then the periodontist will be eliminated as specialists. Certainly the day is on the horizon when the choice to extract a tooth will be as rare as the amputation of an infected limb and considered almost as primitive.

Through our many human pulp studies standards have been established that define the tolerance level of survival for the dental pulp. With these standards, the clinicians now have an understanding as to which procedures are best for the dental pulp and can provide a better quality of clinical practice. Empiricism is losing ground in the field of restorative dentistry. As this area of research continues, more and more "wives' tales" will be gradually eliminated from dental curriculae and hopefully forgotten in the world's dental literature.

In the same sense, similar standards of treatment need to be developed in the field of periodontology. Despite accepted periodontal treatment procedures, the frustrating problem of progression of periodontal disease is still too frequently with us. The knowledge to be gained from such extreme approaches as replantation and root resection will help us zoom in on less obvious, more subtle but critical insidious events.

It is obvious in dealing with the areas of stomatitis and oral cancer that the discipline of immunology is looked upon as our greatest helping hand to solve some of our most difficult problems. At least if etiology is not soon revealed, a greater understanding of many of these odd oral diseases will be helpful in directing future research. Repeatedly some aspect of immunology is reflected in many of the annual reports to follow.

As replantation is a study in extreme to help us gain fundamental knowledge concerning periodontal disease, so in the study of Behcet's syndrome hopefully knowledge will be gained to help us comprehend and treat realistically the less severe diseases such as aphthous stomatitis and periadenitis.

In all our efforts there is an undercurrent of the importance of the "normal." Not only in many dental problems, pulp biology and periodontology, but also in the oral mucosa, the pharynx, larynx, tongue, and temporomandibular joint, studies defining the range of normal are underway. Our profession so frequently has been misled by incorrect published information or the lack of adequate information concerned with the normal aspect of some portion of the head and neck area that such emphasis is duly warranted. It is a very healthy sign that among our staff so much attention is being given to the "normal," indicating a level of suspicion and uncomfortableness concerning our present level of knowledge and understanding.

Certainly from the technical side of dentistry, our clinicians justifiably take their position alongside all the other specialties. In working with cancer, heart, and arthritic patients, the dental clinician has become a recognized member of a team of practitioners who is consulted not only after surgery but prior to and during the procedure in order to provide the best and most satisfying result for the suffering individual, not only from the functional but cosmetic point of view.

In describing our program in a less specific manner, this summary has been held to a few pages. Although every effort has been made to recognize all projects equally, no doubt some bias could have arisen due to the author's emphasis in areas of greatest personal experience.

Report of the Oral Medicine and Surgery Branch
National Institute of Dental Research
Summary Statement

With gradual expansion of the Oral Medicine and Surgery Branch we have attempted to acquire staff that would give emphasis to all the main problem areas of clinical dentistry--caries, periodontal disease, aphthous stomatitis, orthodontics, prosthodontics, endodontics, oral surgery and oral pathology. Although some areas are still under-staffed because of recruitment problems and space we feel that we are finally on the way.

A. Biology of the Human Dental Pulp: In this project there continues an evaluation of the response of the human dental pulp to changes induced by dental drilling procedures and by various restorative and related materials, such as cavity liners. This study has furnished the dental profession with considerable practical information on operative procedures, particularly in regard to optimal cutting speeds, the proper use of coolants, and modifications in technic such as employing liners and varnishes necessary for the safe placement of toxic restorative materials.

Since pulp inflammation following high speed cutting technics is minimal as compared to low speed cutting technics, the incidence of reparative dentin formation is also reduced. Thus, cut dentinal tubules remain completely patent permitting the toxic or irritating products of cements, silicates, and epoxy resins to permeate to the pulp tissue and cause unnecessary and frequently irreversible damage. This lack of reparative dentin formation is creating a formidable problem in restorative dentistry, especially in the field of full mouth rehabilitation where often the entire coronal dentin becomes exposed through full-crown preparation. Experimental drugs designed to reduce sensitivity of teeth (i.e., corticosteroid compounds) and to more effectively seal the dentinal tubules are being sought, as well as drugs and technics to increase the incidence of reparative dentin formation.

When prepared cavities are washed with a steroid formula, containing 1% prednisolone in a vehicle of parachlorophenol, cresatin and gum camphor, before restoration with zinc oxide and eugenol, the pulp response to the cavity preparation is considerably minimized. When the prednisolone is used without the vehicle, the inflammatory response is minimized only 12 days. Also when the same formula is applied to the cavity preparation several days after the full potential of the response has occurred, the resolution period is still shortened.

In an attempt to find more ideal restorative materials, collaborative research is being conducted with various dental manufacturers and the National Bureau of Standards who provide us with experimental restorative materials. Some of the experimental adhesive materials have the potential of supplanting the

commonly used anterior restorative materials such as silicate and acrylic resin. A new alloy composed of gallium-tin-palladium is supposedly superior to dental amalgam. Also a new temporary protective packing (Pharmatec), designed to function both as a temporary filling material and a provisional adhesive agent for crowns and bridges, is being evaluated.

Because the Clinical Center can supply only about 300 human teeth per year for such studies it is necessary to supplement our needs with contracts with several universities and other government facilities.
(See Methods Employed and Major Findings)

In order to better correlate clinical symptoms of pulpitis with histopathologic pulp changes a means of standardizing clinical symptoms with intentional salivary contamination of a cavity preparation has been developed. This will greatly increase the diagnostic ability of the clinician. Another study to determine the rate of movement of bacteria in exposed dentinal tubules is in progress, an area of dental research that has been surprisingly neglected.

Interestingly enough it has become apparent that the leprosy bacillus has a proclivity for the dental pulp of maxillary anterior teeth and can represent the first signs of re-activation of the disease. Such cases of leprosy are being pursued.

Other clinical studies just underway involve the response of the pulp to the removal of cementum and the response of the periodontal ligament and alveolar bone to intentional perforation of previously intact root by endodontic procedures.

In germfree rats several studies have been completed. The healing of experimentally exposed pulps in rodents is primarily dependent on the absence of a microbial flora. This healing process appears not to be altered substantially by the application of conventional or steroid drugs.

B. Clinical and Morphologic Studies of Human Dentition: To characterize the clinical and microscopic manifestations of the deciduous and permanent dentitions in inherited or acquired metabolic disorders has revealed several interesting findings. In hereditary hypophosphatemia, dentinal defects and tubular tracts in the pulp horn region by which microorganisms gain access to the pulp have been demonstrated.

Premature exfoliation of deciduous or permanent teeth has taken on added significance as an ominous diagnostic sign for the physician, pediatrician, and dentist.

The metabolic defect of the odontoblast is assumed to be similar to the defect in metabolic transport proposed for the proximal renal tubular cell such that phosphorus cannot be incorporated into the apatite salts of the dentin. This clearly demonstrates the significance of incorporation of phosphorus into the calcifying dentin, an observation that may be most important from the standpoint of strengthening the tooth substrate and possibly increasing the resistance of the tooth to caries attack by the prophylactic addition of dietary phosphate supplements.

C. Dental Caries: From a research standpoint, rampant caries offers a most favorable opportunity to study the basic factors which activate or control the caries process because the usually prolonged time element in the development of carious lesions is reduced to a minimum.

Clinical studies have indicated that the frequent eating of foods containing sucrose or other fermentable carbohydrates is a very important factor in the etiology of rampant caries. But during the past year an attempt has been made to find foods containing considerable amounts of fermentable carbohydrates but not cariogenic. Although fish protein concentrate contains from 100 to 250 ppm Fluoride, this fluoride probably accounts for only part of the anticariogenic effect, mainly due to the relatively high level of calcium, phosphorus and basic amino acids.

There is a need to develop more non-cariogenic snacks. The demonstration that fish protein concentrates exert a great anticariogenic effect on sucrose suggests that this material may be helpful in not only overcoming human malnutrition but also in the control of caries.

The fluorescent antibody technique enables the microbiologist to examine the actual bacterial flora of dental plaque and tooth sections rather than having to rely on what organisms grow out (and out-grow others) in various artificial media. Since the plaque smears and paraffin tissue sections are stable for staining at a much later date, strict time elements can be eliminated from sample procurement.

D. Studies of Soft Tissue Lesions:

1. Precancerous changes: Human buccal mucosa, although appearing clinically normal, may undergo various changes with the increasing age of the patient. Since the buccal site is frequently biopsied, standards need to be established on normal mucosa to eliminate errors in diagnosis due to the age factor. There is a need for correlation of clinical and histologic changes in the oral mucosa resulting from intrinsic or extrinsic factors. Particular interest is directed towards the keratinizing lesions of a premalignant or closely related nature.

Preliminary studies using tritiated thymidine on oral mucosa involved in verrucous carcinoma have revealed epithelial turnover rates remarkably similar to those believed to be found in normal oral mucosa. Similar studies have been performed on the oral mucosa of patients with Darier's disease. Preliminary results reveal a need for further utilization of this technique in the study of normal as well as disease states involving the oral mucosa.

Detailed clinical and morphologic studies of leukoedema have been carried out with verification of the existence of this clinical entity originally described by Sanstead and Lowe in 1953. Comparison and analysis of histologic materials from their original study in 1953 with our cases revealed the histologic nature of the lesion to be chiefly one of parakeratosis and acanthosis; however, our studies gave no indication that the lesion was, as originally stated, of nutritional origin or that it was in any way premalignant.

A previously undescribed change in the superficial epithelial layers of chronic hyperplastic oral mucosa has been studied. It occurs chiefly in specimens of epulis fissuratum though it may also be seen in irritation fibroma. On the basis of its morphology and histochemical finding of -SH groups, it has been referred to as "keratin pools." This change may be related to a particular environmental agent and may represent an allergic or toxic manifestation.

2. Recurrent aphthous stomatitis: Investigations continue to determine whether a relationship exists between the L-form of an alpha streptococcus in recurrent aphthous ulcers and the etiology of this disease or whether the disease is one of hypersensitivity.

The immunology of these streptococci, streptococcal antigens, streptococcal immune complexes, and antistreptococcal sera are studied in both human and animal cell culture systems. Also, the histocompatibility of aphthous and nonaphthous cells in cell culture using radiated and nonradiated cells are investigated, as well as the role of lymphocytes in host-defense mechanisms. The successful treatment of a patient with immuno-suppressive drug indicates that the etiology of this disease is in part an immune mechanism.

An attempt is being made to develop an accurate diagnostic test based on the presence of specific hypersenstivity in aphthous patients and determine if immunization would be an effective means of treatment.

The laboratory experimental model for aphthous stomatitis is the hypersensitized albino female guinea pig, Hartley strain. Intradermal skin tests with aphthous streptococcus antigen indicate the degree of hypersensitivity and the effectiveness of desensitization procedures.

The repeated intravenous injection of the Eli Lilly Streptococcal vaccine into hypersensitized guinea pigs reduces the skin reaction to the streptococcal cell wall antigen (CHO) indicating some degree of desensitization. Similarly in certain patients the vaccine produced a gradual decreaæ in frequency and intensity of the disease.

Also an effort will be directed toward demonstrating the specific antibodies against the aphthous streptococcus 2A in the circulating lymphocytes of aphthous patients.

3. The Study of Behcet's Syndrome: In this newly initiated program it is very necessary to study the natural history of this syndrome, in an attempt to more closely define its nature, extent and course and at the same time derive information concerning possible etiology and a realistic therapeutic regime for these patients.

In studying these patients emphasis will be placed on lymphocyte transformation, antibodies to various oral and ocular antigens, flocculation and other serologic reactions, cryoglobulins, and immunoglobulins of various body fluids and cultures for microorganisms.

Many serious diseases, often of the connective tissue type, have important oral manifestations. Behcet's syndrome is such an example, since 80 percent of these patients present with aphthous stomatitis. As an adjunct to the above program investigations will proceed on the mechanisms of cryoprecipitation in cryoglobulins, to determine the conformational and other physicochemical and immunochemical properties that affect cryoprecipitation, to find specific causative determinants of cryoprecipitation factors affecting protein solubility and structural aspects of immunoglobulins, and to find a more rational therapeutic approach to cryoglobulinemia.

The isolated cryoglobulin has the solubility characteristics of a euglobulin. Its intrinsic viscosity increases as temperatures increase. Reduction stops cryoprecipitability; reoxidation results in regaining this property but alkylation destroys cryoprecipitability irreversibly.

The study of the immunologic behavior of protein in a wide variety of connective tissue diseases, such as S.L.E. and rheumatoid arthritis brings many important aspects to oral medicine as well.

E. Clinical periodontal studies:

1. By studying the response of the periodontal tissues to tooth reimplantation it is hoped that some knowledge relative to the factors permitting the differentiation of fibroblasts into cementoblasts will be obtained. After reimplantation, the teeth become firmly attached with new alveolar bone formation. In the absence of continued cementum production, periodontal fibers lose their root attachment with eventual exfoliation. New functioning cementoblasts have not been found earlier than 29 days. Similar studies are being carried out on baboons with the intention eventually to substitute artificial teeth for natural teeth.

2. In the treatment of the osseous defects in periodontosis, preliminary results indicate that the autogenous transplantation of developing third molars into the first molar sites can be an effective way of inducing healing of the alveolar bony lesions and in restoring periodontal health.

3. A long-term study of periodontal disease in a stable, adult male population has completed its first year. The occurrence of destructive periodontal disease is being assessed by direct observation on such a population. Examinations will be repeated biannually. A series of preliminary field examinations have been conducted to test the adequacy and reproducibility of the clinical criteria developed for use in this study. The initial series of examinations have been completed but the statistical analysis is incomplete.

4. Children with bizarre gingival lesions not corresponding to any human gingival disease or oral manifestations of any systemic disease are being studied. Their lesions are characterized by severe gingival recession about the labial surfaces of one or more maxillary teeth. The gingival margins in the affected areas are usually ragged and bleeding.

Prior to onset of oral problems, such children experience a sense of deprivation Much anxiety is expressed by the parents as such lesions become the center of family attraction. Gingival self-mutilation becomes a way of handling the anxiety generated by experiencing a loss of dependency needs.

5. The staining morphology of various dental materials in tissue

Problems of identification exist when dental materials are found in oral and superficial gingival tissues microscopically. By intentionally placing various materials into the rat dermis and running a series of staining reactions, identification of some of these extraneous materials when encountered will be more easily recognized.

6. A study to determine the effectiveness of root amputation procedures to prevent progression of pocket formation is underway. The controversy of root amputation versus root planing and curettage has been debated for a number of years. Thus far, teeth treated by root amputation seem to be maintained easier.than those in which curettage is employed. This is a major problem facing the therapist. Many conflicting opinions have been voiced but no attempt at a controlled study has been carried out.

7. The presence of dense cortical plate of bone lining most long-standing infra-bony defects has been implicated in the lack of success of new attachment procedures utilized in clinical periodontal therapy. Some therapists puncture or remove this bony plate but no evidence exists as to whether this exposure of marrow spaces would speed up bone formation with attachment in mind. To study the quantitative and qualitative aspects of healing of bony defects when the cortical plate covering such a defect has been removed is presently in progress.

E. Animal periodontal studies:

It appears that differences in the chemical constituents of saliva can influence calculus formation. The saliva of Holtzman rats fed either a high fat or a high protein diet for 30 days reveals more acid and alkaline phosphatase activity and an increased protein content of the whole mixed saliva.

8. Epileptics since the late 1930's have experienced the side effect of dilantin gingival hyperplasia. Years ago at NIDR considerable research was carried out on rats with no positive results.

Since cats are now recognized as the most suitable animal for producing gingival dilantin hyperplasia, a renewed effort will be made to determine its mode of action.

F. Oral surgery studies:

1. General anesthesia on ambulatory dental patients.

Nitrous oxide was the first general anesthetic used by dentists. Because of its relative safety, no reliable records were kept on morbidity and mortality.

With the advent of sodium pentothal and other intravenous drugs, which were more potent, it became important to study the changes in normal physiology associated with their use. We are constantly improving and refining present methods of anesthesia as well as laying a sound foundation for further research. Our studies have indicated the importance of nitrous oxide and oxygen supplementation and demerol premedication to assist in the control of adverse rises in blood pressure and tachycardia.

2. Post-surgical tissue healing.

To eliminate localized osteitis, the most encountered complication of third molar surgery, neospirin powder is placed in the socket. At this point the efficacy of neospirin has not been exciting.

3. Sectional roentgenographic study of the temporomandibular joint following bilateral osteotomy of the ramus of the mandible.

What happens in the TMJ? Does relapse of the newly established occlusion occur? This investigation will delineate any subjective or objective changes of the condylar head in the glenoid fossa.

4. Evaluation of premedication in conjunction with local anesthesia in oral surgical procedures

Hospitals today are too overcrowded to admit patients just for the benefit of general anesthesia; therefore, different types of premedication drugs are evaluated. Each procedure is done with a different intravenous premedication. It is hoped that the oral surgeon will be another tool for effective pain control.

G. Oral pathology investigations:

1. Submucous fibrosis

Submucous fibrosis is a disease very prevalent in India of unknown etiology but associated with a very high prevalence of oral cancer. It is the intent of this study to determine if chili powders can produce submucous fibrosis in the buccal pouch of hamsters.

2. Betel quid carcinoma

In a study of hamsters' pouches to determine the individual and/or combined roles that calcium hydroxide, tobacco and gambier might play in causing betel quid induced carcinomas, it was found that:

a. Calcium hydroxide caused chemical burns with necrosis and ulceration followed gy regeneration. Three of these animals had atypical epithelial lesions, resembling focal leukoplakia in man. The affected pouches of hamsters treated with calcium hydroxide showed one or more of the following lesions: deposits of calcium, inflammation, fibroblastic proliferation, ulceration, atrophy, hyperplasia, hyperkeratosis, parakeratosis, acanthosis, and cellular atypia.

No squamous cell carcinomas were produced in any of the groups. But it has not yet been ascertained whether the lesions that developed were the final phase of the reaction of the treatment with calcium hydroxide or whether they had the potential of progression to neoplasia. No changes were noted in cheek pouches treated with snuff or starch powder alone. Several hamsters treated with gambier developed minute ulcers with inflammation.

Distinguishing the causative carcinogenic agent in the betel quid chew is important to geographic pathology because of the high incidence of oral carcinoma in the area of the world where quid chewing is prevalent. Until the causative factor is determined public health measures cannot be realistically investigated.

In an effort to modify the epithelium of the hamsters' pouch, two experiments have been initiated to induce liver cirrhosis. Once sufficient liver damage is induced, the cheek pouches will be retreated to determine whether the prevalence for neoplastic change is increased. More and more evidence is accumulating to justify the belief that alcoholics are more prone to oral cancer.

Oral Pharyngeal Development Section: The activities of this Section continue in diversification appropriate to the background and interests of investigators, but each project is oriented to an aspect of development of form and/or function of the mouth, pharynx and larynx.

A new extension is that into embryology, as L. Krames is engaged in study of the neural determinants of the laryngeal skeleton, employing isotope labelling of elements of the neural plate which migrate to form the larynx.

Studies of postnatal development of the rat head skeleton are in stages of publication by M. Baer, J.F. Bosma, and J. Ackerman.

The sequential development of the dental alignment and the facial skeleton in childhood is under epidemiological study by R.D. Christensen, L.F. Mills, and R.L. Christiansen, with observation of differential effects of serial dental extraction upon crowding of incisor teeth. R.D. Christensen, in collaboration with endocrinologists J. Roth and P. Gordon is specifying the acromegalic distortions of facial skeleton and associated soft tissue per cephalometric radiographs. The contribution of orthodontics to correction of skeletal deformities and dental alignment is under demonstration by P. Coccaro in cleft palate children and in a child who had suffered fracture of a mandibular condyle in infancy.

Correlated studies of form and of functions of sensation and of the separate motor actions of feeding and of speech have further evidenced aspects of the intimate association (or relation) between somatic form and neurally governed performance. Utilization of these composite criteria of form, sensation and movements, makes possible a more comprehensive definition of disorders or abnormalities. In some clinical circumstances such as that of "facial hypoplasia" the abnormalities of sensation afford clearest definition of the entity. In others, such as amyotrophic lateral sclerosis, the utilization of dual criteria of motion performance provide useful clues to available or potential motor resources.

The correlations of form and function occasion new questions for investigation. Such, for instance, as the relation between abnormalities of facial form and disorders of smell and taste. Appropriate study is directed toward the neural determinants of somatic form in the facial area, but also to the possibility that both the facial skeleton and the chemosensory mechanisms of this area may reflect (evidence) a primary endocrine determinant. It is clear that the development of form is itself a function, but a function which reflects a composite or cynosure of multiple factors or influencing mechanisms, which contribute in a succession or temporally ordered array in embryonic, fetal, postnatal, and postmature development.

This composite approach of clinical description gives us opportunity of definition of some of the facially malformed children and adults. But these criteria are not yet appropriate to the study of orally, facially, pharyngeally malformed infants, because of lack of anatomical criteria of form, of criteria of sensory-guided motor function, and of criteria of subjective indications of, or response to sensory stimuli.

303

Thus, at present, we are limited to the retrospections of the clinical histories of the disabilities in infancy of feeding and of respiratory functions in children, who may be identified syndromically. And, reciprocally, we must study the dysphagic and respiratory-impaired infants by standard routines of still and cine photography and radiography at appropriate sequences during their development until defined by criteria which are identified in older children as known or specific syndromes of disorders.

Serial No. NIDR-65 (c) (63)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Histopathology of Oral Mucous Membrane

Previous Serial Number: NIDR-54 (c)

Principal Investigator: Dr. H.O. Archard

Other Investigators: None

Cooperating Units: National Cancer Institute, Dermatology Branch

Man Years:

Total:	3/4
Professional:	3/4
Other:	0

Project Description:

Objectives:

1. Correlation of clinical and histologic changes in oral mucosa resulting from intrinsic or extrinsic factors. Particular interest is directed towards the keratinizing lesions of a premalignant or closely related nature.

2. Identification of significant variables related to these clinical and morphologic changes.

Methods Employed:

1. Patients with oral keratinizing lesions are screened and a detailed environmental history obtained. This data is supplemented with clinical photographic records where possible.

2. Representative biopsy material is obtained and processed by routine histologic techniques. Repeat biopsies are obtained where possible to delineate the pathogenesis of the disease process.

Major Findings:

1. Leukoedema, as originally described by Sandstead and Lowe in 1953, has been re-defined in the light of careful clinical and morphologic studies of some of Sandstead's original material and several additional cases followed at the Clinical Center for a period of time. There was no indication that the lesion was, as originally proposed, of nutritional origin or that it was premalignant.

2. A previously undescribed change in the superficial epithelial layers of chronic hyperplastic oral mucosa has been studied clinically, morphologically, and histochemically. It occurs chiefly in specimens of epulis fissuratum though it may also be seen in irritation fibromas and probably other pathologic mucosal tissue. On the basis of its morphology and the histochemical finding of -SH groups, it has been referred to as "kertin pools" and appears most commonly in chronically inflamed hyperplastic mucosa, particularly that associated with dentures (i.e., epulis fissuratum).

Significance to Dental Research:

1. It is now possible to separate histologically the condition known as leukoedema of the oral mucosa from otherclinical white lesions, some of which are of a premalignant or malignant nature.

2. The identification of the unusual "keratin pooling" in the superficial epithelium in chronic hyperplastic oral mucosa has considerable significance since this change may be related to a particular environmental agent (e.g., acrylic material of dentures) and may represent an allergic or toxic manifestation.

3. Biodynamic studies of oral mucosa in various reactive and neoplastic (premalignant and malignant) states are potentially most significant. Such studies will provide information regarding the cellular activity of these various human oral lesions and indicate which morphologic changes have greatest prognostic value.

4. The identification of specific morphologic alterations of the human oral mucosa in relation to particular environmental agents still occupies a pre-eminent position in applied clinical research. Prevention of most of these mucosal diseases is predicated on the identification and control of these environmental factors.

Proposed Course of Project:

1. The spectrum of keratinizing human oral lesions should be studied longitudinally for both the clinical and light microscopic

changes which occur. Each case should be approached from the standpoint of obtaining a detailed environmental analysis in conjunction with a properly documented record of the morphologic changes in order to demonstrate the clinicopathologic progression of the disease. Such studies, however, will require enhanced support in the form of improved technical services and facilities.

2. Biodynamic studies of selected cases with keratinizing hyperplastic or neoplastic oral lesions should be undertaken to assess the kinetics of these lesions. A sufficient number of cases will be needed to establish a significant norm for each of the various types of keratinizing oral lesions.

3. Appropriate correlative studies of these lesions should be pursued employing such established techniques as electron microscopy and/or histochemistry.

Part B

Publications:

1. Archard, H.O., Carlson, K.P., and Stanley, H.R.: "Leukoedema of the human oral mucosa." Oral Surg. 25: 717-728, May, 1968.

2. Archard, H.O.: Chapters for "Dermatology in General Medicine" edited by Fitzpatrick, Van Scott, et al: Chap. 28) "Biology of the human oral integument" (accepted for publication). Chap. 29) "Common stomatologic disorders" (in preparation). Chap. 30) "Oral manifestations of systemic diseases" (in preparation).

Serial No. NIDR-66 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Clinical and morphologic studies of the human dentition in metabolic diseases, either acquired or inherited.

Previous Serial Number: NIDR-55 (c)

Principal Investigator: Dr. H.O. Archard

Other Investigators: None

Cooperating Units: Section of Child Neurology, National Institute of Neurological Diseases and Blindness; Metabolic Diseases Branch, National Institute of Arthritis and Metabolic Diseases; Clinical Endocrinology Branch, National Heart Institute

Man Years:

Total:	1/4
Professional:	1/4
Other:	0

Project Description:

Objectives:

1. To characterize the clinical manifestations of the deciduous and permanent dentitions in inherited or acquired metabolic disorders, particularly those currently under investigation by the various Institutes of the National Institutes of Health.

2. To characterize the gross and light microscopic alterations in the deciduous and permanent teeth from patients with inherited or acquired metabolic disorders.

Methods Employed:

1. Patients under treatment for metabolic disorders are frequently seen in the Dental Services Branch of the Clinical Center. With the cooperation of the attending physician and dentist such patients are seen on a referral basis, and details of the clinical history, oral examination, and dental roentgenographic studies are incorporated into the patients' permanent records.

2. Arrangements are made for the proper histologic study of any teeth that are to be removed or are indicated for eventual removal. The teeth may be studied by routine decalcification, sectioning, and staining, or by preparation of ground sections, depending on the nature of the metabolic disease process or the requirements of the tissue to be studied.

Major Findings:

1. Certain known inherited metabolic disorders have recently been recognized to present characteristic clinical and morphologic dental manifestations. We have characterized in detail both the clinical and morphologic features of the deciduous teeth in hereditary hypophosphatemia (vitamin D-resistant rickets) and clearly demonstrated previously undescribed dentinal defects and tubular tracts in the pulp horn region by which microorganisms gain access to the pulp of otherwise normal appearing teeth.

2. Teeth from various metabolic diseases such as hypophosphatasia, pseudohypoparathyroidism, Hurler's syndrome, diabetes insipidus, etc., are in process of being examined, and clinical and morphologic changes will be reported when sufficient case material is available.

Significance to Dental Research:

1. The characterization of the morphologic changes in the dentin of the deciduous teeth of patients with hereditary hypophosphatemia has provided a basis for understanding the unusual clinical dental manifestations in this disease.

2. The metabolic defect of the odontoblast in hypophosphatemia is assumed to be similar to the defect in metabolic transport proposed for the proximal renal tubular cell such that phosphorus cannot be incorporated into the apatite salts of the dentin. This clearly demonstrates the significance of incorporation of phosphorus into the calcifying dentin, an observation that may be most important from the standpoint of strengthening the tooth substrate.

3. Premature exfoliation of deciduous or permanent teeth has taken on added significance as an ominous diagnostic sign for the physician, pediatrician, and dentist.

Proposed Course of Project:

1. Further collection of tooth specimens (both deciduous and permanent) from patients with known metabolic diseases will be undertaken.

Part B

Publications:

1. Archard, H.O. and Witkop, C.J.: "Hereditary hypophosphatemia (vitamin D-resistant rickets) presenting primary dental manifestations. Oral Surg. 22: 184-193, August, 1966.

310

Serial No. NIDR-67 (c) (59)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Studies on the Etiology and Treatment of Periodontal Diseases in Children and Adolescents

Previous Serial Number: NIDR-56 (c)

Principal Investigator: Dr. P.N. Baer

Other Investigators: None

Cooperating Units: Dr. H. Hoffman, National Institute of Mental Health

Man Years:

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives:

1. To investigate the etiology of bizarre gingival lesions which occur in young children. The lesions are characterized by severe gingival recession about the labial surfaces of one or more maxillary teeth. The gingival margins in the affected areas are ragged and bleeding.

Methods Employed:

1. Children with bizarre gingival lesions which do not correspond to any known gingival disease or oral manifestation of any known systemic disease were studied. These patients were examined by a physician who, with the aid of proper laboratory tests ruled out the presence of a systemic disease. The patients and their parents then receive psychiatric interviews. Emphasis was placed on interviewing the parents as a couple, and, when indicated, individually.

Major Findings:

1. Prior to the onset of the oral problems, each of the affected children was experiencing a sense of deprivation.

2. In all cases a documented organic gingival lesion occurred during the time each child was experiencing the stress associated with the frustration of his dependency needs.

3. A marked shift in the family dynamics occurred. Much anxiety was generated by the occurrence of oral pathology and each child became the center of his environment.

4. It was hypothesized that at a time of extreme stress these children had organic oral pathology which significantly altered their environment. Their dependency needs were then met, albeit in a regressive way. They aggravated these lesions by excoriating and denuding their gingiva. This habit of gingival mutilation became a way of handling the anxiety generated by experiencing a loss of dependency needs.

Significance to Dental Research:

Studies of this nature may provide new insights into the etiology of certain types of periodontal lesions.

Proposed Course of Project:

Further investigations into other etiologic factors which may play a role in periodontal diseases in children is being pursued.

Part B not included

Serial No. NIDR-68 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Studies on Experimental Calculus Formation

Previous Serial Number: NIDR-58 (c)

Principal Investigator: Dr. P.N. Baer

Other Investigators: Dr. P.H. Keyes, Dr. I. Zipkin, Mr. C. White, Mr. N. Mantel, and Mr. G.R. Hawkins

Cooperating Units: None

Man Years:

Total:	1-1/4
Professional:	1/4
Other:	1

Project Description:

Subproject A:

Objectives:

To investigate whether the differences noted in calculus formation between Holtzman rats obtained from two different sources could be explained, in part, by the differences in the chemical constituents of the saliva.

Methods Employed:

Forty-four female, weanling Holtzman rats, 22 from the NIH breeding colony and 22 from the Holtzman Laboratories, Madison, Wisconsin, were used in this study. Each group was divided and fed either a high fat or a high protein diet, known to encourage calculus formation. At the end of 30 days, saliva was collected and chemically analyzed for acid phosphatase, alkaline phosphatase, protein, total carbohydrate, phosphorus, calcium, and rate of flow. The animals were then sacrificed, decapitated, the heads defleshed, and the molar teeth scored for calculus.

Major Findings:

1. Calculus deposition was greater in the Holtzman rat obtained from the NIH colony than from the colony obtained from Madison, Wisconsin.

2. The NIH Holtzman rats maintained on high fat and high protein diets showed more acid and alkaline phosphatase activity and an increased protein content of the whole mixed saliva, as compared to the Holtzman rat fed the same diet from the Holtzman Laboratories, Madison, Wisconsin.

Subproject B:

Objectives:

To investigate the effect on calculus formation of: (a) antibiotic administration, (b) housing a substrain that is relatively susceptible to calculus formation with one that is relatively resistant, and (c) diet.

Methods Employed:

To investigate the effects of an antibiotic and intermixing of the two substrains on calculus formation, 15 NIH and 15 Wisconsin weanling female rats were caged separately, two animals per cage, while another 15 NIH and 15 Wisconsin rats were housed together, two of each strain to a cage. They were fed a high protein calculus-inducing diet for 30 days. In addition, 15 NIH and 15 Wisconsin rats, housed separately, were fed a high protein diet which contained 100 mg. of penicillin G per kilogram of diet. To investigate the effect of composition and consistency of diet on calculus formation, five groups, consisting of 15 animals per group of weanling female NIH Holtzman rats, were placed on the following dietary regimes: (1) a high protein diet for 60 days; (2) a high protein diet for 30 days and then Purina chow soaked in water, for 30 days; (3) a high protein diet for 30 days and Purina chow, pellet form, for 30 days; (4) Purina chow, pellet form, for 60 days; (5) Purina chow, mush consistency for 60 days. At the end of the experimental periods the animals were sacrificed, and the molar teeth scared for calculus.

Major Findings:

1. Factors involved in calculus formation were shown to be transmissible in rats fed a high protein, calculus-inducing diet.

2. Penicillin incorporated in the calculus-inducing diet significantly reduced the amount of calculus formed.

3. Housing a strain of rat which was relatively resistant to calculus formation with one that was relatively susceptible significantly increased the amount of calculus deposition in the more resistant strain.

4. A nutritionally balanced, commercially available diet significantly reduced calculus formation to even a greater degree than penicillin.

Significance to Dental Research:

Calculus is believed to be an important etiologic factor in periodontal disease; all knowledge which can be obtained concerning its formation should be of major interest.

Proposed Course of Project:

Further studies on the effects of nutritional and transmissibility factors as they affect calculus formation will be pursued.

Part B

Publications:

1. Baer, P.N., Hawkins, G.R., Wells, H., Mantel, N., and Zipkin, I.: Studies on Experimental Calculus Formation in the Rat. XI. Relation to Diet and Selected Salivary Constituents. J. Periodont. 38: 323-329, July-August, 1967.

2. Baer, P.N., Keyes, P.H., and White, C.L.: Studies on Experimental Calculus Formation in the Rat. XII. On the Transmissibility of Factors Affecting Calculus. J. Periodont. (in press)

3. Baer, P.N.: Use of Laboratory Animals for Calculus Studies. Proc. N. Y. Acad. Sciences (in press)

Serial No. NIDR-69 (c) (68)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: The Effects of Blastomycosis on Oral Tissue

Previous Serial Number: None

Principal Investigator: Dr. W. A. Bell

Other Investigators: Drs. G. E. Garrington and J. W. Gamble

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description:

Objectives:

1. To study the disease pattern of North American blastomycosis in oral structures and differentiate it from those of other oral granuloma.

2. To evaluate histologically the tissue penetration pattern of the blastomycosis organism.

3. To evaluate treatment methods used on the patients reviewed.

Methods Employed:

Patient records from the National Institutes of Health of patients with oral blastomycosis are being reviewed along with submitted histologic material.

Major Findings:

The clinical and histologic material is still being gathered and evaluated at this time.

Significance to Dental Research:

1. Large numbers of case reports are needed to properly evaluate diagnosis, treatment, and probable disease pattern.

2. Mycotic diseases of the mouth and para-oral structures are seen with increasing frequency by pathologists.

3. The importance of patient histories will enable the practicing physician or dentist to possibly link environmental or occupational hazards.

Proposed Course of the Project:

It is intended to have at least eight or nine cases with documented follow-up. New cases will be added to the above group as they are submitted.

Part B not included

Serial No. NIDR-70 (c) (68)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: The Staining Morphology of Various Dental Materials in Tissue

Previous Serial Number: None

Principal Investigator: Dr. W. A. Bell

Other Investigators: Dr. G. E. Garrington

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description:

Objectives:

1. To provide a histologic demonstration of the various dental materials as seen when surgically introduced into the dermis.

2. To evaluate tissue response for each given substance.

3. To evaluate the staining characteristics for determining the identity of the embedded substance.

Methods Employed:

Various materials have been gathered which include silver amalgam particiles, gold dust, gutta percha, carborundum dust, zinc phosphate cement dust, Kerr's root canal sealer, silicate cement, enamel dust, steel filings, calculus particles, diamond dust particles, and pumice.

Major Findings:

The materials needed for this project are being gathered at this time.

Significance to Dental Research:

1. When evaluating autoradiography studies with hemotoxylin and eosin-staining techniques, some foreign-body material can be accidentally introduced. This study would possibly help identify some of these extraneous materials.

2. When evaluating dental or para-oral structures histologically, pigments are often encountered whose exact nature cannot be determined morphologically.

Proposed Course of Project:

Various dental materials will be surgically placed in the extremities of experimental animals and the staining characteristics of each substance will be noted.

Part B not included

Serial No. NIDR-71 (c) (68)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: A Measure of the Effectiveness of Root Amputation as a Means of Slowing Pocket Progression

Previous Serial Number: None

Principal Investigator: Dr. A.F. Binderman

Other Investigators: Dr. T. Lundy

Cooperating Units: None

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

1. To determine the effectiveness of root amputation procedures in the long-term change in rate of progression of pocket formation into the furcation areas of teeth.

2. To determine, from clinical staining procedures, differences in abilities to maintain root-amputated teeth hygienically, in comparison to a group of root-planed, curetted teeth with similar involvement in the same patient.

Methods Employed:

1. During a two-year period of time, patients exhibiting similar involvements of furcation areas on a molar on both the right and left sides of the arch have been utilized.

2. On one side, dependent upon which furcations are involved, under xylocaine anesthesia, a root is removed. On the other side, also under xylocaine anesthesia, thorough root planing and curettage are accomplished. Both sides have a dressing placed for two weeks. At this time the dressings are removed and both sides are cavitroned on exposed tooth surface to remove all particles of pack, desquamated epithelial cells, etc.

3. Previous to the above, all teeth have been thoroughly scaled and the patients instructed on home care procedures (modified Stillman's technique utilizing a Broxodent electric toothbrush, Stim-U-Dents, and use of a Lactona interdental stimulator), and have had a careful occlusal adjustment. Thus, each patient commences with only the variable of home maintenance to act on the rate of pocket formation.

4. At two-month intervals, radiographs and clinical photos of the operated areas, after the patient has utilized a crest dye tablet, are taken. Plaque formation around the two teeth is noted in this manner, and pocket depth is recorded. Patients will remain under observation for a period of two years.

Major Findings:

1. Thus far, teeth treated by root amputation seem to be more easily maintainable than those in which curettage is employed. These are only, however, very short-term observations, and inasmuch as the study is a long-term result, are, as yet, of little value.

Significance to Dental Research:

1. No long-term studies of tooth survival after varying therapeutic regimes have been done in bi- and tri-furcation involvements. This is a major problem facing the therapist. Many conflicting opinions have been voiced regarding root amputations as a long-term solution to this problem, but no attempt at a controlled study has been yet made. This study will be important, therefore, in evaluating long-term prognosis, on the basis of a two-year maintenance trend, and thus help to determine if root amputation is actually an effective procedure.

Proposed Course of Project:

Dependent upon results of this study, if root amputation is determined to be a valid therapeutic procedure, the study will branch into the restorative aspect of the question. Series of teeth with one or two roots removed will be restored with full crowns, giving them proper contour relationships with the hard and soft supporting tissues. These will be compared to unrestored, root-amputated teeth, in terms of plaque deposits, pocket formation, and mobility, over a course of two years.

Part B not included

Serial No. NIDR-72 (c) (68)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Studies in the Healing of Alveolar Bone in Dogs.

Previous Serial Number: None

Principal Investigator: Dr. A.F. Binderman

Other Investigators: Drs. P.M. Lightbody and P.N. Baer

Cooperating Units: None

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

1. To study the quantitative and qualitative aspects of the healing of a bony defect when the cortical plate covering such a defect has been removed.

2. To compare these qualitative and quantitative aspects with those of defects in which the cortical covering has not been removed.

3. To determine the causes of variations in the healing rates in the two aforementioned variable conditions.

Methods Employed:

1. Six dogs, each placed under IV nembutal anesthesia, were subjected to the extraction of maxillary and mandibular left and right second bicuspids. The right side remained as a control.

On the left side, utilizing a high speed #2 round bur, numerous perforations were made through the cortical plate of the socket, into the adjacent cancellous bone, in order to expose and give access to

the marrow cavities within this bone, effectively removing the cortical plate. The sockets on both the left and right sides were closed by the drawing over and suturing of the overlying soft tissues. The animals were then placed on a liquid diet for one week.

2. At intervals of 3, 5, 7, 15, 30, and 90 days, the dogs were, and are, being sacrificed and four block sections are taken. These will include the extraction site and one adjacent tooth on either side.

3. Radiographs are then taken on occlusal films as one means of comparison of new bone formation.

4. The block sections are then prepared for sectioning and staining (H&E, Mallory trichrome).

There is no patient material.

Major Findings:

1. Examinations of radiographs show them to be not sensitive enough to pick up slight variations in degrees of bony fill and calcification, at least to the extent that variations exist within the framework of this study.

2. Histologic sections are being prepared at the present time.

Significance to Dental Research:

The presence of a dense, cortical plate of bone lining most long-standing, infra-bony defects has been implicated in the lack of success of new attachment procedures utilized in clinical periodontal therapy. In order to more predictable achieve their desired results, many therapists have been puncturing or removing this bony plate as a part of their procedure. No evidence exists, however, as to whether this exposure of marrow spaces would speed up bone formation rate, or would, in fact, retard it, because of the inflammatory reaction that could be caused by the manipulation of the bone. This study will measure the relative responses accompanying such a therapeutic variable, determining whether it is justified or not.

Proposed Course of Project:

Dependent upon histologic results, clinical application of this decorticalization will be evaluated in patients undergoing periodontal therapy in the Clinical Center.

In a series of lesions specifically indicated for new attachment

procedures, decorticalization will be applied. After following their course by monthly radiographs taken with utilization of the x-ray grid over the film (to measure degree of new bony attachment), at six months, the areas will be re-entered and re-photographed to determine what their course has been. (re-entry will be necessary anyway, inasmuch as recontouring of bone to its desired architectural form must be done in re-attachment procedures in the vast majority of cases).

A trend in clinical predictability may then be offered by study of these cases; clinical comparisons are at best unlikely in the same arch, as two lesions of comparable architectural arrangement are rarely found in one mouth.

Part B not included

Serial No. NIDR-73 (c) (68)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Study of Behcet's Syndrome

Previous Serial Number: None

Principal Investigator: Dr. Norman A. Cummings

Other Investigators: Dr. Thomas Francis, Dr. Donald Bergsma, Dr. John Graef, Dr. Thomas Minas, Dr. Vernon Wong, Dr. John Decker, Dr. Daryll DeVivo and Dr. Richard Epstein

Cooperating Units: NIAMD, Arthritis and Rheumatism Branch; NINDB, Ophthamology Branch; NINDB, Medical Neurology Branch; NIMH, Laboratory of Clinical Science; NCI, Dermatology Branch

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

1. To recruit and select patients for clinical study with Behcet's Syndrome and related systemic diseases with aphthous stomatitis or other serious oral manifestations.

2. To study the natural history of these syndromes, in an attempt to more closely define their nature extent and course.

3. To correlate histopathologic, cultural and chemical findings with clinical observations in order to derive information concerning possible etiology of these conditions.

4. To derive a successful and clinically substantiated therapeutic regime for these patients.

Methods Employed:

1. Patients are screened for admission by outpatient examination or perusal of referral records.

2. Admitted patients have detailed history and physical examinations performed; tests on serum and cerebrospinal fluid are carried out for a variety of studies. Included are: (1) lymphocyte transformation, (2) antibodies to various oral and ocular antigens, (3) flocculation and other serologic reactions, (4) cultures for bacteria, fungi, PPLO and viruses, (5) cryoglobulins, and (6) immunoglobulins of various body fluids.

3. All patients have careful attention paid to nervous system, ocular, and psychiatric status.

4. Tissue biopsy is performed, with cultures, of indicated oral, skin, eye and genital lesions.

5. X-ray examination of sacro-iliac joints is carried out.

6. Other clinical and laboratory parameters as indicated are obtained when necessary.

Major Findings:

This phase has just begun in late 1967. Careful correlation and evaluation of data is not yet ready, and would be premature.

Significance to Dental Research:

Many serious diseases, often connective tissue diseases, have important manifestations in the mouth. Behcet's Syndrome is a major example, since 80% of these patient's present with aphthous stomatitis. This syndrome is common in Mediterranean countries and may be more prevalent in the U.S.A. than previously thought. The Syndrome itself can be malignant and may carry a mortality of over 10%. It can result in blindness, neurologic disability, and long years of pain and illness.

Besides studying the course, etiology and potential therapy of Behcet's Syndrome, this project attempts to consider those broader aspects of serious disease states which have important oral manifestations. This seems particularly true of connective tissue diseases (such as Behcet's), and thus their study may serve as a link between the basic research and clinical aspects of both internal medicine and dentistry.

Proposed Course of Project:

As above, under Objectives and Methods Employed.

Part B not included

Serial No. NIDR-74 (c) (68)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Mechanisms of Cryoprecipitation in Cryoglobulins

Previous Serial Number: None

Principal Investigator: Dr. Norman A. Cummings

Other Investigators: Drs. Martin D. Lidsky and Henry Metzger

Cooperating Units: Houston Veteran's Administration Hospital, Baylor University College of Medicine, Arthritis Research Section; NIAMD, Arthritis and Rheumatism Branch

Man Years:

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives:

1. To determine the conformational and other physiochemical and immunochemical properties that affect cryoprecipitation, and to study analytically what departure from normal amino acid content or arrangement leads to this change.

2. To find specific causative determinants of cryoprecipitation, and compare these with information from parallel studies on cryoglobulins derived from other species.

3. To interpret the evidence in terms of factors affecting protein solubility and structural aspects of immunoglobulins.

4. To gain better insight into the possible relationship between protein solubility and certain connective tissue diseases.

5. To lead to a more rational therapeutic approach to cryoglobulinemia.

Methods Employed:

1. Cryoglobulins are isolated from the sera of affected patients by repeated cold precipitation and washing. Purity is evaluated by analytical ultracentrifugal, electrophoretic, and solubility studies, as well as immunochemical techniques.

2. The isolated cryoglobulins are examined in different media to study conformational changes at different temperatures. Experiments are performed using viscometry, titration, diffusion, optical rotatory dispersion, ultra-violet spectroscopy, and analytical ultracentrifugation.

3. In parallel experiments, the globulin molecule is digested into various fragments and chains in order to more closely approximate that portion of the molecule wherein the properties responsible for cryoprecipitability reside.

4. Peptide mapping of smaller fragments are compared to analogous molecular fragments from normal IgG. Amino acid composition by spetrophotometric and chemical techniques is determined.

5. Relationships between structure and solubility is considered in light of the above data, with regards to (a) cryoprecipitability; (b) cryoglobulins from other species; (c) structure and function of immunoglobulins; (d) problems of protein solubility in terms of thermodynamic parameters, and (e) possible relationship to connective tissue diseases.

Major Findings:

1. The isolated cryoglobulin has the solubility characteristics of a euglobulin, and satisfies criteria of purity immunoelectrophoresis, cellulose acetate electrophoresis, gel filtration, and analytical ultracentrifugation. It has and $S_{20,w}$ of 6.61; solubility is a linear function of both temperature and ionic strength.

2. The characteristics of molecules precipitating at various temperatures are the same by all physicochemical criteria determined.

3. Intrinsic viscosity of the cryoglobulin increases significantly as temperatures increase from 25° to 37° C. in 1.0μ buffer; similar changes do not occur in normal IgG. No aggregation is detected under these conditions.

4. Reduction stops cryoprecipitability; reoxidation results in regaining this property. Alkylation after reductions destroys cryoprecipitability irreversibly.

5. Peptic digestion results in a 5S fragment which retains over 30% of cryoprecipitability, implying that the C-terminal half of the heavy chains are not necessary.

Further reduction and alkylation reveals that the light chains do not cryoprecipitate; the N-terminal half of the heavy chains are insoluble in aqueous media.

6. Spectrophotometric titration data indicate a decrease in the tyrosine content of cryoglobulin compared to normal IgG, while tryptophan is unchanged. These data are confirmed by standard amino acid analyses.

Significance to Dental Research:

This work studies the relationshop between protein solubility and structure with regards to temperature. It offers an opportunity to explore the phenomenon of solubility as well as the primary to tertiary structure and immunochemical behavior of normal and pathologic immunoglobulins.

In addition to gaining further insight into immunologic behavior of proteins, a subject in itself important with respect to many aspects of oral medicine, the project deals with a protein found to be present in a wide variety of connective tissue diseases. These diseases, such as S.L.E., often have important oral manifestations; the relationship of protein precipitates to pathological lesions, such as found in rheumatoid arthritis, etc., is yet to be fully explored from a physicochemical point of view, whether those lesions are generalized or located specifically in the mouth.

Proposed Course of Project:

As outlined above under Objectives and Methods.

Part B

Publications:

1. Cummings, N.A., Kuff, E.L., and Sober, H.A.: Examination of Magnesium Binding to Serum Proteins by Ultracentrifugal Analysis. Analytical Biochemistry 22: 108, 1968.

330

Serial No. NIDR-75 (c) (58)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: General Anesthesia on Ambulatory Dental Patients

Previous Serial Number: NIDR-56 (c)

Principal Investigator: Dr. E.J. Driscoll

Other Investigators: Dr. P. Lightbody

Cooperating Units: Dr. C.L. Hebert, Anesthesiology Department, Clinical Center, Dr. C. Battig, Biomedical Engineering and Instrumentation Branch, DRS

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

This is a report of a recently reactivated project. However, since almost all of the personnel, both professional and ancillary had to be trained or retrained and since the research endeavor has only recently assumed a normal operating facility, this report is necessarily concise and sketchy.

Objectives:

To study the changes in normal physiology which are caused by or associated with the general anesthetics which are used by oral surgeons on ambulatory dental patients.

Our original study became operational in 1958 and was temporarily interrupted for 18 months in 1966.

Methods Employed:

1. Full mouth extractions are selected rather than random oral surgical procedures, so that the surgical trauma could be standardized. The teeth are extracted in quadrants, and the entire operation is systematized for over-all time of procedure and traumatic experience.

2. A multi-channel polygraph is employed and the physiologic data being recorded are: pulse, blood pressure, arterial O_2 saturation, respiratory phenomena, cortical brain activity and the electrical activity of the heart (EKG). Likewise recorded are such important technical data as amount of drug used in units of time, length of operation, duration of sleep, mental alertness on awakening, age, race, sex, emotional status in relation to the conduct of anesthesia and post-anesthetic sequelae (headache, nausea and vomiting, depression, and hiccoughs).

Patient Material:

So far we have reported on over 1200 operations in utmost detail. We have no significant numbers to add to this total in this past year's limited experience.

Significance to Dental Research:

1. One of the first surgical procedures ever performed under general anesthesia was a dental extraction. The short dental procedure was found to be very suitable to the early attempt to accomplish painless surgery on the unconscious subject. Dentistry was, therefore, closely identified historically with the early development of general surgical anesthesia.

   Nitrous Oxide was the first general anesthetic used by dentists and it actually survived the early "trial and error" experience. In the early 20th Century it was the only general anesthetic used by the majority of dental specialists for procedures on ambulatory patients.

   Very little was known about the physiological behavior associated with the use of this gas; but, because of its relative safety and the fact that practically no reliable records were kept on morbidity and mortality, the true story was never known, fully appreciated or for that matter even questioned.

2. With the advent in the late 1930's of Sodium Pentothal and other intravenous drugs, which were also found very applicable to dental anesthesiology, a change in philosophy was inevitable. The drugs were much more potent and since they could easily kill or cause serious morbidity, a much more cautious attitude was necessary.

3. Our research team as well as others interested in this area of investigation are diligently searching the literature and other sources for information regarding newly discovered anesthetic agents which might be safer and more effective. These drugs are being and will continue to be produced; but, without these baseline data on physiologic response, which are the results of our previous studies, it would be difficult to impossible to evaluate them.

4. We are constantly studying the possibility for improvement and refinement of present methods of anesthesia, as well as laying a sound foundation for future anesthesiology research. An example of refinement in methods is indicated in our studies of N_2O and O_2 supplementation and demerol premedication, both of which have been shown to assist in the control of adverse rises in blood pressure and to a lesser degree tachycardia.

Proposed Course of Project:

We have established the broad baselines of performance on all anesthetic agents and combinations thereof, which are presently utilized by oral surgeons and we are now ready to examine some of these physiological parameters in detail and to attempt practical clinical interpretations.

Changes of significant proportions have been shown to occur in such vital physiological areas as blood pressure and pulse, and it is, therefore, extremely important that the causes of these peripheral cardiovascular responses be investigated in depth. For example, if an increase in cardiac output is found to attend these blood pressure and pulse elevations, this is a far more favorable finding in terms of patient safety, than if a decrease in cardiac output is demonstrated. If the latter is the case, then the pulse and pressure elevations are potentially a far more serious finding.

Other very fertile areas for future investigation stem naturally from our past demonstration of deviations from normal physiology in the anesthetized state. Various abnormalities in the EKG have been consistently shown to occur in this type of anesthesia. A more detailed investigation is planned to study these changes in the electrical conduction system of the heart.

Part B included: Yes

Part B

Honors and Awards relating to this project:

1. Horace Wells Award 196/ - For outstanding research in dental anesthesiology.

Serial No. NIDR-76 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Immunologic, Biochemical and Microbiological Studies in Recurrent Aphthous Stomatitis

Previous Serial Number: NIDR-59 (c)

Principal Investigator: Dr. T.C. Francis

Other Investigators: Dr. H.R. Stanley, Dr. J.F. Bosma, Dr. E.A. Graykowski

Cooperating Units: Division of Biologics Standards, Dr. M.F. Barile

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives:

1. To study the clinical aspects of aphthous stomatitis.

2. To study the microbiologic and immunologic relationship of Streptococcus sanguis and other related streptococci in this disease.

3. To determine if a relationship exists between the presence of the L-form of an alpha streptococcus in recurrent aphthous ulcers and the etiology of this disease.

4. To study the immunology of these streptococci, streptococcal antigens, streptococcal immune complexes, and antistreptococcal sera in both human and animal cell sulture systems.

5. To study the histocompatibility of aphthous and nonaphthous cells in cell sulture using radiated and nonradiated cells.

6. To investigate the role of the lymphocyte in host-defense mechanisms.

7. To record daily long term records on these aphthous patients in order to evaluate the frequency, severity of lesions and, their possible relation to stress, trauma, drug therapy, or other factors.

8. To collaborate with Dr. Cummings in the study of Behcet's Disease.

Methods Employed:

1. The following tests are performed: red blood count, white blood count, the differential, Wintrobe indices, sedimentation rate, complement fixation, urinalysis, serum iron, total serum proteins, C-reactive protein, anti-streptolysin O titers, Strep. MG titers.

2. The oral lesions of all patients are biopsied, part of this tissue is sent to the histopathology laboratory and part is prepared for bacterial and viral studies.

3. Thoroughness of examination of the microbiologic and histopathologic aspects in a few selected patients are emphasized.

4. Lymphocyte transformation studies are now in progress to determine the in vitro effect of strep antigens on human peripheral lymphocytes and animal lymphoid cells. This will include patient groups consisting of: (1) normals, (2) Aphthous Stomatitis, (3) Behcet's Disease, (4) post streptococcal illness, and (5) other.

5. The evaluation of our long term patient records (kept daily by the patients) has been analyzed by computer analysis. The results indicate that the frequency of lesion occurrance, the number of lesions, and their severity is a completely random process and is not correlated to any other clinical variable recorded.

Patient Material:

Patients are selected from those on record from the earlier stomatitis studies of the Dental Institute and those being referred at the present time to the Dental Services Branch, NIDR. About 40 patients comprise the study group.

Major Findings:

1. Final findings indicate that peripheral leucocytes from aphthous and normal patients show no significant difference in their response to the streptococcus sanguis 2A isolated by Dr. Graykowski.

2. A more basic finding is that human pathogenic streptococci stimulate human cell cultures significantly more than non pathogenic strains and conversly, animal pathogenic streptococci stimulate animla lymphocytes significantly more than either non pathogens or human pathogenic strains.

3. The treatment of a single aphthous patient with an immunosuppressive drug resulted in a period of remission from lesions.

Significance to Dental Research:

The study attempts to explain the etiology of Aphthous Stomatitis and possibly discover more suitable methods of controlling the disease. Other allied projects investigate the nature of streptococcal hypersensitivity in humans, and the nature of recurrent ulcerative stomatitis as seen in Behcet's Disease.

Proposed Course of Project:

The general course of the project will continue along the lines of investigation already outlined in this report.

The treatment strongly indicated that (1) the etiology of this disease is in part an immune mechanism and (2) based on this and other data, a broader project study may be indicated to further evaluate the therapeutic efficacy of these drugs. Several other patients treated in this manner in other clinics have produced similar results.

Beginning in September, the prinicpal investigator, Dr. Francis, will begin a two year period of training. Arrangements have been made to maintain routine folow-up care for the clinical patients on this study. It is hoped that many of the laboratory projects already in progress can also be continued.

Part B

Publications:

1. Francis, T.C., and Archard, H.O.: Nasopalatine duct cyst with epidermoid features; report of case, J. Oral Surg., 25: 1967.

2. Barile, M.F. and Francis, T.C.: Streptococcus sanguis in the pathogenesis of recurrent aphthous stomatitis. In Protoplasts, Spheroplasts, and L Forms. Guze, L. (Ed.) Williams and Wilkins Co., Baltimore. In press.

3. Francis, T.C., Oppenheim, J.J., Barile, M.F.: Lymphocyte transformation by streptococcal antigens in guinea pigs and man. In Proceedings of the Third Annual Leukocyte Conference, 1967. Riecke, W. (Ed.) (Appleton-Century, Crofts, N.Y.) In press.

Serial No. NIDR-77 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Osteosarcoma and Chondrosarcoma of the Jaws

Previous Serial Number: NIDR-60 (c)

Principal Investigator: Dr. G.E. Garrington

Other Investigators: None

Cooperating Units: Armed Forces Institute of Pathology

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

1. To define the behavioral patterns of these tumors in the jaws and compare this to behavior in other bones.

2. To better define the clinical and radiographic character of these tumors, thereby aiding in earlier diagnosis.

3. To evaluate therapeutic measures.

4. To determine prognosis in relation to therapy.

Methods Employed:

Fifty-six cases of osteosarcoma and 36 cases of chondrosarcoma primary in the maxilla or mandible were selected because they appeared to satisfy the established criteria of these tumors. Complete follow-up information including hospital and clinic records, dental and skeletal roentgenograms and clinical photographs, was obtained. Additional and more current information was obtained from the patient's physician and/or dentist, from patients still living,

and from death certificates and autopsy protocols of those patients who died. Histologic slides were carefully examined, and the collected materials were evaluated and subjected to statistical analysis leading to a correlation of clinical, histologic and radiographic findings with diagnosis as well as to a correlation of therapy to survival.

Major Findings:

1. Osteosarcoma and Chondrosarcoma do apparently behave somewhat differently in the jaws in relation to each other than they do in other bones. Specifically, Osteosarcoma appears to have a better prognosis and Chondrosarcoma a worse prognosis in the jaws than in other bones. The survival rate for Chondrosarcoma in other bones is much better than for Osteosarcoma; this appears to be reversed for the tumors in the jaws.

2. Radical resection appears to offer the best hope for cure for either tumor.

3. Tumors in the symphysis of the mandible have the best prognosis and those in the maxillary antrum have the worst prognosis.

4. An important early finding is roentgenographic evidence of a symmetrically widened periodontal membrane space with maintenance of an intact lamina dura.

5. The histologic degree of malignancy does not appear to be related to prognosis.

6. Prognosis is apparently somewhat better than has been previously reported.

Significance to Dental Research:

1. This study should provide better definition of a rather poorly characterized group of tumors.

2. The differences in behavior and prognosis that are pointed out should provide the clinician with a firmer basis for deciding what therapy to use.

3. There are some subtle indications in this study that there may be a sex difference in the distribution at least of the osteosarcomas. If this is true, it should provide another bit of data for the building of a case for hormonal influences on tumorigenesis and control.

Proposed Course of Project:

The collection and evaluation of materials on osteosarcoma and chondrosarcoma have been completed. It is now intended to begin study of another group of poorly understood primary bone tumors: the myxomas and myxocarcomas. The present intent is to follow basically the same pattern of study that was used for the osteosarcomas and chondrosarcomas; to try to determine whether the myxomatous group of tumors is in fact an entity or is perhaps a more primitive form of the osteogenic group; and to determine whether the myxomatous tumors primary in bone are similar to, or different from, the odontogenic myxomatous tumors.

Part B

Publications:

1. Garrington, G.E., Scofield, H.H., Cornyu, J. C. and Hooker, S.P.: Ostoesarcoma of the Jaws. Cancer 20: 377-391, March, 1967.

340

Serial No. NIDR-78 (c) (67)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Leprosy involving the Dental Pulp

Previous Serial Number: None

Principal Investigator: Dr. G. E. Garrington

Other Investigators: Dr. M. C. Crump

Cooperating Units: U.S.P.H.S. Hospital, Carville, La.; U.S.P.H.S. Hospital, San Francisco, Calif.; U.S.P.H.S.Outpatient Clinic, San Pedro, Calif.; Leonard Wood Memorial (Cebu City, Phillipines, Leprosy Laboratory)

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

1. To determine the morphologic manifestations of dental pulpal infection due to leprosy.

2. To establish a basis for determination of the best form of therapy (endodontic versus exodontic).

Methods Employed:

Extracted teeth from patients with leprosy have been collected from the National Institutes of Health, the Carville Hospital, and the San Pedro Outpatient Clinic. These have been evaluated for pulpal infection by leprosy bacilli.

They are presently being evaluated for viability of organisms by personnel of the Leprosy Service at the San Francisco Hospital who have devised a method of determining viability of leprosy bacilli

in paraffin sections. Contact has been made with the Leprosy Laboratory at Cebu City in the Phillippines, through Dr. Chapman Binford, Director of the Leonard Wood Memorial. The dentist at Cebu City has consented to submit extracted teeth from there. This should provide a source of teeth from persons with tuberculoid leprosy, which is desirable, because most of the teeth obtained from this country are from patients with lepromatous leprosy. The teeth and any surrounding soft or bony tissues removed are to be studied histologically and histochemically for infection and alterations.

Major Findings:

1. In the teeth examined thus far, it is apparent that leprosy can and does involve the dental pulp to the point of producing pulp degeneration and dental symptoms.

2. It appears that pulpal involvement may be a manifestation of active disease.

3. Mycobacterium leprae can invade the dentinal tubules of unrestored, caries-free teeth.

Significance to Dental Research:

The effect of pulpal infection by leprosy bacilli is not presently known. This study will define these effects and provide for better clinical evaluation of dental pain in leprosy patients.

Proposed Course of Project:

Teeth will continue to be collected, principally from Cebu City, in order to determine the extent of pulpal involvement due to tuberculoid leprosy as compared to lepromatous leprosy. An effort will be made to assess the relative merits of extraction versus endodontics in cases of pulpitis due to leprosy.

Part B

Publications:

1. G. E. Garrington and M. C. Crump: Pulp Death in a Patient with Lepromatous Leprosy, Oral Surg., Oral Med., Oral Path.; 25:427-434, 1968.

Serial No. NIDR-79 (c) (67)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Dilantin Gingival Hyperplasia

Previous Serial Number: None

Principal Investigator: Dr. G.E. Garrington

Other Investigators: Dr. Harold Fullmer

Cooperating Units: V. A. Hospital, Washington, D.C.; U.S. Army Institute of Dental Research; possibly, the Johns Hopkins Hospital; Epilepsy Clinic and Parke-Davis Company will be involved, but this is not yet definite.

Man Years:

Total:	1/4
Professional:	1/4
Other:	0

Project Description:

Objectives:

1. To determine the mechanism by which dilantin causes gingival hyperplasia.

2. To study the morphologic and developmental character of the process in humans and in experimental animals.

3. To assay enzymatic activity in dilantin hyperplastic tissue and compare it with the activity in non-hyperplastic tissue from epileptics on dilantin, with tissue from epileptics not on dilantin, with tissues from normal patients and with animal tissue from cats medicated with dilantin.

4. To determine whether there are changes in vascularity or gingival circulation in dilantin hyperplastic tissues as opposed to normal tissues.

5. To assay dilantin levels in gingival tissue.

Methods Employed:

The project is in the period of planning and initiation, now. The following methods are anticipated:
1. Approximately 50 patients, principally from the V.A. Hospital, with dilantin gingival hyperplasia of varying degree will be biopsied. A small group of patients on dilantin with no clinical hyperplasia will be biopsied. A small group of epileptic patients not on dilantin will be biopsied. And a small group of non-epileptic patients will be biopsied. Portions of the excised tissue will be fixed in formalin for routine histology and special histochemistry. Another portion of excised tissue will be immediately frozen and used for enzyme studies. A third portion is tentatively proposed for use in assaying dilantin levels in the tissue itself. This latter is dependent upon the cooperation of Parke-Davis Company in performing the assays.

2. Dilantin will be administered to cats to promote gingival hyperplasia and basically the same studies will be done that are proposed in (1) above to determine the comparability of dilantin gingival hyperplasia in cats to that in humans.

3. A small number of cats will be injected with a silicone rubber preparation that is useful for tracing circulation. This method has been found useful at the U.S.A.I.D.R. for demonstrating microvasculature. The object is to compare minute circulation in the hyperplastic tissues with that of non-hyperplastic basically normal tissues.

Major Findings:

The project is in the initial stages at present.

Significance to Dental Research:

1. As currently projected, the major significant finding is hoped to be a clue as to what initiates the hyperplasia. This is intended as a step toward determining what leads to tissue proliferations including tumors.

2. The study of enzymatic activity in the gingival specimens should add to the rather meager current knowledge on that subject. It may be that this will add to knowledge of periodontal disease as well. One specific enzyme to be assayed is collagenase, thereby perhaps shedding some light on the relationship it may have to gingival disease.

Proposed Course of Project:

As stated previously, the study is now in the initial stages. The course proposed is to follow the methods outlined in the "Methods Employed" section above. Following completion of the study it is proposed to follow-up any leads that may be developed regarding tissue proliferation and tumorigenesis.

Part B

Not included.

Serial No. NIDR-80 (c) (62)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Microbiological Phase of Rampant Caries Study

Previous Serial Number: NIDR-61 (c)

Principal Investigator: Mrs. S. A. Geis

Other Investigators: Drs. R. M. Stephan and R. J. Fitzgerald

Cooperating Units: None

Man Years:

Total:	1-1/4
Professional:	1
Other:	1/4

Project Description:

Objectives:

The objective of this project is to identify and classify human oral streptococci and to determine whether or not specific strains of streptococci can be identified as etiologic agents in dental caries. The flourescent antibody technique is employed to identify specific streptococci strains in histologic sections of carious teeth and in the mixed culture of dental plaque smears.

Methods Employed:

Laboratory research into possible streptococcal causes of rampant caries may be classified into three phases:

1. The production, testing and purification of diagnostic antisera to oral streptococci.
2. Testing streptococcal antisera to cultures, plaque smears and histologic sections of teeth taken from patients.
3. Animal studies.

Rabbits already highly immunized to human oral streptococci were kept on antisera production with weekly booster shots of heat-killed

vaccines and periodic bleedings to provide a constant source of fresh antisera. New antisera were developed to four organisms freshly isolated from new rampant caries patients. All of the organisms possessed metabolic indicators of possible cariogenic activity; they produced dextran from sucrose, produced iodophyllic intracellular polysaccharide and fermented mannitol and sorbitol. One had colony morphology like the cariogenic rat streptococcus strain FA1. One was serologically related to Dr. Zinner's cariogenic strain BHT (which is supposed to be closely related to rat strain FA1) as well as our own strains PH4, 2KD, 1DD, BY and a Lancefield Group A. This cross reaction necessitated careful investigation and absorption of several antisera.

The crude and absorbed antisera were tested against samples of dental plaque smears in an effort to obtain a pattern of antigenic importance of the streptococcal components of plaque and to ascertain the relative presence of these organisms in plaque from persons with and without severe dental caries problems.

Streptococcus strain RC2 was introduced into a tank of germ-free rats to study its cariogenic ability. The organism was isolated from a child with rampant caries and is serologically reciprocally cross-reactive with the Zinner strain BHT.

Two studies on the effects of feeding germ-free rats diets 585V and 2000V were done in order to have control comparison animals for our gnotobiotic studies.

Tissue studies were completed on the germ-free experiment involving mono-infection of rats with human Streptococcus mitis strain PH4. The results were analyzed and reported.

Major Findings:

Interesting serological cross reactions were found to exist between streptococci isolated from rampant caries patients and some of the streptococci isolated by Dr. Zinner in Miami. Since the Zinner BHT organism is cariogenic and related to the rat cariogenic streptococcus strain FA1 it was necessary to determine the relationship, natural occurrence and cariogenicity of the serologically related strain RC2. With careful absorptions it was possible to remove cross reactions from the RC2 serum and from the BHT, PH4, 2KD, 1DD and BY sera. The absorbed RC2 serum was reactive with 20 to 50% of the cocci in the plaque smears tested, which was a lower reactivity than the crude serum. The absorbed BHT serum had a much lower reactivity with plaque cocci than the crude serum. Many plaque samples that were cultured on mitis-salivarius agar and smeared on slides for the

fluorescent antibody stain had many cocci reacting to BHT serum but failed to grow out either BHT or RC2 type colonies in culture.

The Rampant caries streptococcus strain KB1 isolate that has colony morphology similar to BHT and FA1 colony morphology is not serologically related to either strain. Reactivity of cocci in plaque smears to KB1 antiserum varies widely.

Streptococcus strain RC2 was allowed to become established in the mouths of 7 germ-free rats for three months. Gross examination at sacrifice and examination of the 1/2 of each head defleshed and dried revealed minimal caries formation. Present evidence indicates that streptococcus strain RC2 is not cariogenic.

Tissue sections of gnotobiotic rats infected with streptococcus strain PH4 failed to explain the small white lesions present on the lung surfaces of the rats at autopsy. All the rats had emphysema and calcification in the kidneys but the PH4 streptococcus was not implicated in the pathology. The PH4 gnotobiotic rats had minimal caries formation.

Two germ-free control studies were done but examination of only one has been completed. All six rats on diet 585V and and all six rats on diet 2000V had fractures of the molars but no caries. Although the large contaminating bacillus was present in the sulci among food particles there was no invasion of the fractures or the dentin tubules. The bacillus was present in the lumena of the stomach and intestines but was absent in all other tissues. All of the rats on both diet 585V and 2000V had emphysema and at autopsy one rat on diet 585V and one rat on diet 2000V had tiny, white lesions on the surface of the lungs. The contaminating bacillus was not implicated in the lesions or the emphysema. The rats on diet 585V did not have any areas of calcification in the kidneys but two of the six rats on diet 2000V had small, scattered areas of calcification. Only gross autopsy data is available for the uncontaminated germ-free control study. No lung lesions were present in the four rats on diet 585V; no gross fractures or caries were seen in the teeth. All five rats on diet 2000V had small, white nodules on the surfaces of the lungs. none of the rats on diet 2000V had gross fractures or carious lesions in the teeth.

Significance to Dental Research:

The fluorescent antibody technique developed in this study enables the microbiologist to examine the actual bacterial flora of dental plaque and tooth sections rather than having to rely on what organisms grow out (and out-grow others) in various artificial media. This can be

of major significance in epidemiological studies of dental caries. Since the plaque smears and paraffin-fixed tissue sections are stable for staining at a much later date, strict time elements can be eliminated from sample procurement.

Proposed Course of Project:

It is planned to continue to develop purified antisera to additional strains of streptococci isolated from carious areas of human teeth. The fluorescent antibody technique, using new and presently developed antisera, will be applied to future clinical and animal studies and in epidemiological surveys of the caries problem. Attempts will be made to recognize cultural and metabolic characteristics of cariogenic streptococci in addition to those factors already theorized to be associated with cariogenic activity. Work will continue on the problem of establishing probable cariogenic human streptococci in test animals.

Part B not included

Serial No. NIDR-81 (c) (65)
1. Oral Medicine and Surgery
3. San Francisco, California

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Immunological Studies in Recurrent Aphthous Stomatitis

Previous Serial Number: NIDR-62 (c)

Principal Investigator: Dr. E. A. Graykowski

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

1. To develop further evidence that recurrent aphthous stomatitis is a hypersensitivity disease.

2. To develop an immunologic test for diagnosing aphthous patients in an inactive period.

3. To determine if immunization is an effective means of treatment in this disease.

Methods Employed:

1. The following tests are performed on aphthous patients admitted to the study: Red blood cell count, hemoglobin, hematocrit, sedimentation rate, VDRL complement fixation test, total serum proteins and albumin-globulin ratio, serum electrophoresis, and urinalysis.

2. The oral lesions are biopsied; part of the tissue specimen is sent for histopathologic diagnosis, part is utilized in procedures to detect cellular antibodies.

3. Blood is drawn from patients at periodic intervals in the course of their disease to obtain circulating lymphocytes for use in the Jerne plaque test which was designed to detect lymphocyte antibodies against the aphthous streptococcus 2A.

4. The laboratory experimental model for aphthous stomatitis is the hypersensitized albino female guinea pig, Hartley strain. Various desensitization procedures are performed utilizing these animals, such as the injection of intravenous streptococcal vaccines and immunosuppressant drugs. Intradermal skin tests with the aphthous streptococcus antigens are used to indicate the degree of hypersensitivity and the effectiveness of desensitization procedures.

5. A detailed analysis of the aphthous streptococcus antigens is being performed to determine the specific antigenic component for use in diagnostic tests and possibly the treatment of patients.

6. Patients are selected from those referred to the NIDR Unit at the PHS Hospital in San Francisco from military bases and the private practitioners of medicine and dentistry in the area. A study group of approximately 50 patients is planned.

Major Findings:

1. Animal studies have indicated that hypersensitivity to the antigens of the alpha streptococcus isolated from recurrent aphthae is an important factor in the development of these lesions. Positive skin tests (delayed type hypersensitivity) to these antigens are obtained in patients with aphthous stomatitis but not in control individuals. The degree of the skin test reaction is directly proportionate to the severity of the disease in the patient tested.

2. The injection of the Eli Lilly intravenous streptococcal vaccine into hypersensitized guinea pigs over a period of several weeks reduces the skin reaction to the streptococcal cell wall antigen (CHO), indicating some degree of desensitization.

3. The use of the Lilly intravenous streptococcal vaccine in two patients with the severe form of recurrent aphthous stomatitis, periodenitis, produced a gradual decrease in the frequency and intensity of the mucosal lesions with complete cessation of the lesions after a latent period of approximately 6 months after the last injection of the vaccine. One patient has remained free of symptoms for over 2 years and the other for 1 year.

Significance to Dental Research:

The presence of transitional L-forms of an alpha streptococcus in large numbers and in pure culture from numerous lesions, their persistence in lesions in one patient for at least five months, the associated bacteremia during exacerbation, and the recovery of a stable L-form from tissue during remission may be significant. These findings suggest that a relationship exists between the L-form of the streptococcus and the pathogenesis of recurrent aphthae. The finding of a skin hypersensitivity to the suspected causative agent of aphthous stomatitis (L-form of an alpha streptococcus) may prove to be a very important factor in establishing the etiology of this condition and in differentiating it from the other stomatitides.

The apparent desensitization or immunization of two patients preventing the recurrence of mucosal lesions for a long period of time indicates that this may be an effective long-lasting treatment of this disease.

Proposed Course of Project:

A major effort will be made to develop an accurate diagnostic test for aphthous patients. The investigation will be directed toward demonstrating the presence of specific antibodies against the aphthous streptococcus 2A in the circulating lymphocytes of aphthous patients.

An attempt will be made to find a more acceptable means of immunizing patients other than the intravenous administration of a vaccine. The role of the immunosuppressant drugs in creating tolerance to bacterial antigens will be investigated.

Part B

Publications:

1. Graykowski, E.A.: Treatment of Oral Ulcerations, Modern Treatment 4:555-571, May 1967.

2. Barile, M.F., Francis, T.C., and Graykowski, E.A.: Streptococcus sanguis in the Pathogenesis of Recurrent Aphthous Stomatitis. In Guze, L., (Ed.): Protoplasts, Spheroplasts and L-forms, Baltimore, Md., Williams & Wilkins Co., 1968, pp. 444-456.

Serial No. NIDR-82 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Betel Quid Carcinogenesis

Previous Serial Number: NIDR-63 (c)

Principal Investigator: Dr. J. E. Hamner, III

Other Investigators: None

Cooperating Unit: Dr. L. J. Dunham, National Cancer Institute

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives:

1. To determine the individual and/or combined roles that calcium hydroxide, tobacco, and gambier may play in causing betel quid induced carcinomas.

2. To induce squamous cell carcinoma in the hamster cheek pouch, using various betel quids.

3. To determine which of the betel quid ingredients are the causative agent.

4. To relate these findings to the high incidence of oral squamous cell carcinoma in the betel quid chewing areas of the world.

Methods Employed:

Eight groups of male and female hamsters totaling 54 animals were given one of the following treatments: (a) gambier alone, (b) snuff alone, (c) starch powder alone, (d) calcium hydroxide alone, (e) equal parts of calcium hydroxide and gambier, (f) equal parts

of calcium hydroxide and snuff, (g) calcium hydroxide in the morning and snuff in the afternoon, and (h) calcium hydroxide in the morning and starch powder in the afternoon. Hamsters were 3-1/2 to 4-1/2 weeks old when treatment began. A Vienna nasal speculum was used for the applications which were placed in the check pouches five days each week. The hamsters were sacrificed when moribund, or were taken for examination after spontaneous death. Autopsies were performed on each animal and histologic sections prepared.

Major Findings:

1. Calcium hydroxide caused chemical burns with necrosis and ulceration followed by regeneration. Three of these animals had atypical epithelial lesions, resembling focal leukoplakia in man. The affected pouches of hamsters treated with calcium hydroxide showed one or more of the following findings: deposits of calcium, inflammation, giant cells, and fibroblastic proliferation in the lamina propria; and inflammation, ulceration, atrophy, hyperplasia, hyperkeratosis, parakeratosis, acanthosis, and cellular atypia in the epithelium.

2. No squamous cell carcinomas were produced in any of the groups.

3. No changes were noted in the cheek pouches treated with snuff or starch powder alone.

4. Two of 14 hamsters treated with gambier developed minute ulcers with inflammation.

Significance to Dental Research:

1. Distinguishing the causative carcinogenic agent in the betel quid chew is important to geographical pathology because of the high incidence of oral carcinoma in the area of the world where betel quid chewing is so prevalent, namely southeast Asia and India.

2. Until the causative factor or factors are determined, Public Health measures and population education cannot be instigated to reduce the high mortality from oral squamous cell carcinoma in southeast Asia.

Proposed Course of Project:

It cannot be ascertained from this experiment whether the lesions that developed were the final phase of the reaction to the treatment with calcium hydroxide, or whether they had the potential of progression to neoplasia. Studies are planned to increase the contact

with the calcium hydroxide and to modify the environment afforded by the hamster.

In an effort to modify the epithelium of the hamsters' buccal pouch mucosa, two experiments have been started to gradually induce cirrhosis. Twenty hamsters have been maintained on 25% ethylalcohol in their drinking water for 4 months. Twenty other hamsters have had 0.5 ml. injections of 10% CCl_4 in olive oil intradermally for 4 months (1 injection per week). These will continue for 6 months, then liver biopsies will be done to ascertain the presence of cirrhosis.

Once sufficient liver damage is induced, the cheek pouches will be treated with calcium hydroxide, similar to the original experiment.

Part B

Publications:

1. Dunham, L.J., Muir, C.S. and Hamner, J.E., III; Epithelial atypia in hamster cheek pouches treated repeatedly with calcium hydroxide. Brit. J. Cancer 20:588-593, Sept. 1966.

Serial No. NIDR-83 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Benign Fibro-osseous Lesions of the Maxilla and Mandible

Previous Serial Number: NIDR-64 (c)

Principal Investigator: Dr. J.E. Hamner, III

Other Investigators: None

Cooperating Units: Captain H. H. Scofield (DC) USN, Armed Forces Institute of Pathology

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

1. To devise a classification system for benign fibro-osseous lesions found in the jaws from the vast wealth of case material in the files of the A.F.I.P., using a thorough clinico-pathological correlation and accurate follow-up of patients.

2. To correlate the pathogenesis, radiographic picture, clinical behavior, and microscopic features of these lesions (ossifying fibroma, cementifying fibroma, cemento-ossifying fibroma, central fibroma, desmo-plastic fibroma, active ossifying fibroma, giant cell tumor, aneurysmal bone cyst, hyperparathyroidism lesion, monostotic fibrous dysplasia, and familial fibrous dysplasia) by a detailed study.

3. To determine the tissue of origin of these lesions.

Methods Employed:

A complete listing of cases with the above diagnoses was compiled

by the A.F.I.P. Case histories, radiographs, and microscopic material on each case was reviewed. Special stains were used when indicated, and all slides were examined under polarized light. Follow-up forms were mailed to both the attending dentist or physician and the patient. This major project is still in progress and is quite lengthy to complete.

One portion of the project was a histochemical study to determine the tissue of origin of these lesions. Forty-two cases diagnosed as either hyperparathyroidism, aneurysmal bone cyst, giant cell tumor, familial fibrous dysplasia, monostotic fibrous dysplasia, cementifying fibroma, ossifying fibroma, cemento-ossifying fibroma, or active ossifying fibroma were chosen from the main A.F.I.P. listing of these cases. Sections were cut and stained by the following methods: (a) Hematoxylin and eosin, (b) monopersulfate-aldehyde fuchsin-Halmi for oxytalan fibers, (c) Rinehart for mucopolysaccharide and collagen fibers, and (d) Taenzer-Unna orcein for elastic fibers. The H and H sections were also studied under polarized light.

Major Findings:

1. Ossifying fibroma, cementifying fibroma, and cemento-ossifying fibroma arise from the periodontal membrane.

2. Ossifying fibroma can also arise from medullary bone, as do the remainder of the fibro-osseous lesions of the jaws.

3. Oxytalan fibers may occur in most benign fibro-osseous lesions of the jaws, regardless of their tissue of origin, provided that mature collagen fibers are present in the lesion.

4. Inasmuch as oxytalan fibers and pre-elastic fibers cannot be distinguished with present histochemical methods, the demonstration of fibrous elements stained with the oxytalan fiber method does not constitute conclusive evidence of odontogenic origin of the tumor.

5. The birefringence pattern under polarized light does serve as an excellent differential for diagnosis. Fibrous dysplasia gives a random irregular birefringence, indicative of woven bone, whereas; the other fibro-osseous lesions manifested birefringence as parallel light and dark bands, indicative of the varying degrees of lamellar bone formation.

Significance to Dental Research:

1. Fibro-osseous lesions may arise from the periodontal membrane

and the medullary bone when found in the jaws.

2. Oxytalan fibers have again been found in a pathologic condition and have been shown to have a direct relationship to mature collagen fibers.

3. Polarized light has been confirmed as an excellent tool for differential diagnosis for the pathologist.

Proposed Course of Project:

The major portion of this project involving review of the listed cases is almost complete. Approximately 625 cases are well documented and acceptable to be included in the final analysis. The follow-up information requires time to complete. When this part of the project is completed, the final conclusions can be drawn.

Part B

Publications:

1. Hamner, J.E., III and Fullmer, H.M.: Oxytalan fibers in benign fibro-osseous jaw lesions, Arch. Path. 82: 35-39, July, 1966.

2. Hamner, J.E., III, Scofield, H.H., and Conryn, J.: Benign fibro-osseous jaw lesion of periodontal membrane origin: An analysis of 249 cases, Cancer (accepted for publication).

Serial No. NIDR-84 (c) (68)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Submucous Fibrosis

Previous Serial Number: None

Principal Investigator: Dr. James E. Hamner, III

Other Investigator: None

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

1. To determine if chili powders can produce submucous fibrosis in the buccal pouch of hamsters.

2. To compare the effects of chili powders from two states in India (Kerala and Gujarat).

Methods Employed:

Twenty hamsters were given Kerala chili powder via a Vienna nasal speculum in their right buccal cheek three times per week. Biopsies were taken after 1, 2, 4, 6, 8, and 12 months of the treatment, and the tissue specimens were stained with hematoxylin and eosin, Rinehart's stain and van Gieson's stain.

Major Findings:

The experiment is in progress. No findings yet.

Significance to Dental Research:

Submucous fibrosis is an oral disease peculiar to India. The fibrosis affects the buccal mucosa, tongue, palate, and pharynx, causing extreme difficulty in swallowing and eating. Severe cases can be fatal. There is a strong possibility that this is a premalignant condition. Capsaicin (in chili powder) has been suggested as the possible etiologic factor. It is hoped to cause this disease in animals to prove its etiology and give a model for its continued study.

Proposed Course of Project:

The project is now at its halfway mark toward completion. Biopsies of the chili-treated cheek pouches will continue to be taken at the specified intervals. Beginning at the six month mark, tissue specimens will be studied using the electron microscope, as well as the light microscopy procedures.

Of no changes are evident in the pouch mucosa after twelve months treatment, the same treatment will be continued for another year.

Part B not included

Serial No. NIDR-85 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Dental Pulp and Periodontal Studies in Germfree and Conventional Laboratory Rats

Previous Serial Number: NIDR-65 (c)

Principal Investigator: Dr. S. Kakehashi

Other Investigators: Drs. H. R. Stanley and R. J. Fitzgerald

Cooperating Units: None

Man Years:

Total:	1/4
Professional:	1/4
Other:	0

Project Description:

Objectives:

To study the comparative healing of pulp and surgical wounds of periodontal tissues in germfree and conventional rats and how the healing potential can be modified.

Methods Employed:

A total of 56 Fisher rats, consisting of germfree and conventional control animals were surgerized to expose the previously intact pulp of the maxillary first molars. The exposed pulps were treated with corticosteroid and/or a mixture of prednisolone, camphor, methyl cresyl acetate and parachlorophenol which was sealed in place with a temporary filling material. Appropriate control groups were established. Animals were killed at intervals from 1 to 72 days postoperatively. Serial sections of the surgerized tooth were stained with hematolylin and eosin, Masson's trichrome, Giemsa and Brown and Brenn stains.

Major Findings:

1. The conventional animals, regardless of treatment, showed an immediate, severe inflammatory response which quickly led to total pulpal necrosis.

2. The germfree animals showed a minimal inflammatory response followed by a reparative process with complete dentinal bridging. The several modalities of treatment used appeared to be of no value.

Significance to Dental Research:

This study indicates that healing of experimentally exposed dental pulps in rodents is primarily dependent on the absence of a microbial flora. This healing process appears not to be altered by corticosteroid treatment.

Proposed Course of Project:

The effects of surgical wound healing are being studied in a germ-free system.

Part B

Publications:

1. Kakehashi, S., Stanley, H.R., and Fitzgerald, R.J.: The Exposed Germfree Pulp - The Effects of Topical Corticosteriod Medication and Restoration. In press.

Serial No. NIDR-86 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: A Long Term Study of Periodontal Disease in a Stable, Adult, Male Population

Previous Serial Number: NIDR-66 (c)

Principal Investigators: Drs. S. Kakehashi and N. W. Littleton

Other Investigators: None

Cooperating Units: District of Columbia Fire Department

Man Years:

Total:	1-3/4
Professional:	1
Other:	3/4

Project Description:

Objectives:

The purpose of this investigation is to study the initiation and progression of periodontal disease on a long term basis in a stable, adult, male population. The occurrence of periodontal disease is to be studied with regard to selected intraoral factors of suspected etiologic importance.

Methods Employed:

The occurence of destructive periodontal disease is being assessed by direct observation of volunteers of the D. C. Fire Department. Examinations are to be repeated biannually and changes in the initiation and progression of the disease are to be related to the following factors: (1) Gingivitis, (2) Debris, (3) Calculus, (4) Overhangs and Caries, (5) Interproximal Contacts, (6) Mobility, and (7) Occlusion, (a) Centric Permaturities, (b) Balancing Contacts.

Major Findings:

The initial series of examinations conducted on 581 volunteers from the District of Columbia Fire Department have been completed. To date, the statistical analysis of the data obtained from these field examinations is incomplete.

Significance to Dental Research:

The relationship between the various clinical signs of periodontal diseases has not been adequately described. Limitations associated with the clinical and cross-sectional epidemiological study of periodontal diseases, a chronic and progressive process, are well recognized. Consequently, the need for a long term study is not only indicated but appears to be the only method by which this information can be obtained.

Proposed Course of Project:

The second series of field examinations are to commence in September 1968. In the interim, statistical analysis of the data obtained from the initial (cross sectional) examination will be completed.

Part B not included

Serial No. NIDR-87 (c) (67)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Post-surgical Tissue Healing

Previous Serial Number: NIDR-67 (c)

Principal Investigator: Dr. P.M. Lightbody

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

To correlate age of patient, type of impacted tooth, systemic condition of patient and surgical trauma with post-operative localized osteitis. To study the effect of topical antibiotic administration on post-extraction healing.

Methods Employed:

Patients with bilateral impacted mandibular third molars are used. Age of patient, type of impaction, surgical procedure and medication in extraction wound are recorded. On one side no medication is used, and on the opposite side, neosporin powder is placed in the wound. Patients are observed until asymptomatic and healing of each side is compared.

Major Findings:

Thus far, the incidence of localized osteitis has not diminished on the side where medication is used as compared with the control side. The study is too young to report any findings.

Significance to Dental Research:

To try to eliminate localized osteitis, the most frequently encountered complication of third molar surgery.

Proposed Course of Project:

A minimum of 500 impacted teeth are to be removed and results compared. Approximately 75 have been done. Approximately 200 new cases of mandibular impacted third molars are completed.

Part B not included

Serial No. NIDR-88 (c) (67)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Sectional Roentgenographic Study of the Temporomandibular Joint Following Bilateral Osteotomy of the Ramus of the Mandible

Previous Serial Number: NIDR-68 (c)

Principal Investigator: Dr. P. M. Lightbody

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1/4
Professional:	1/4
Other:	0

Project Description:

Objectives:

Long term follow-up of patients subjected to bilateral osteotomy of the mandible is being done to evaluate the following:

1) Any recurrence or relapse of the newly established occlusion.

2) Any subjective or objective changes in the T.M.J.

3) Any x-ray changes such as morphology change of condylar head, change in position of condylar head in glenoid fossa in open and closed positions.

4) Any correlation between type of malocclusion and facial deformity correction and surgical procedure used.

5) Any correlation between timing of auxillary procedures, such as orthodontics, prosthetics and operative dentistry and surgical procedure.

Methods Employed:

Patients who have malocclusions or facial deformities that can be corrected by surgery are admitted to the Clinical Center and the operative procedure selected by the oral surgeon is carried out. Preoperative records include articulated models, pre- and post-operative Panorex x-ray, lateral and posterior-anterior head plates in open and closed position and lateral tomograms of the T.M.J. in open and closed position. Postoperative lateral and posterior-anterior head plates are then taken at 6 months, 1 year and each successive year for a minimum of 5 years. Lateral tomograms are taken at a 1 year postoperative. Clinical examination and evaluation is carried out each time the patient reports for x-rays.

Major Findings:

Approximately 15 patients are over 3 years postoperative. There are some minimal x-ray changes as seen in tomographic examination, however, these are not correlated to clinical findings. There have been no relapses or recurrences.

Significance to Dental Research:

There is some question of what happens in the temporomandibular joint following bilateral osteotomy of the mandible. There is a need for a longitudinal study of this, both from a clinical evaluation and x-ray examination.

There are no longitudinal studies reported that deal with follow-up of patients who have had bilateral osteotomies of the mandible. Possibly different surgical approaches should be used for varying types of malocclusions.

Proposed Course of Project:

It is intended to have at least 50 of these cases with a documented follow-up.

Part B not included

Serial No. NIDR-89 (c) (67)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Evaluation of Premedication in Conjunction with Local Anesthesia in Oral Surgical Procedures

Previous Serial Number: NIDR-69 (c)

Principal Investigator: Dr. P. M. Lightbody

Other Investigator: None

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

To evaluate which premedicating drugs have the most potential for use on oral surgical patients being treated under local anesthesia.

Methods Employed:

Each patient has at least four separate oral surgical procedures done. Each procedure is done with a different premedication. The drug is given intravenously and is titrated for each patient. Vital signs are monitored prior to, during, and after the procedure and compared. Subjective evaluation of each drug is also evaluated.

Major Findings:

The study is too young to report any findings.

Significance to Dental Research:

To give the oral surgeon another tool for effective pain control and to evaluate which drug is best suited for this purpose.

There are many patients who require oral surgical procedures who do not have the benefit of general anesthesia. In many geographic locations in the country general anesthesia is not practiced on ambulatory patients. Hospitals today are too overcrowded to admit patients just for the benefit of general anesthesia; therefore, different types of premedication drugs are being employed.

Proposed Course of Project:

A minimum of 150 patients are to have at least four surgical procedures and results of each drug tabulated.

Part B not included

370

Serial No. NIDR-90 (c) (68)
1. Oral Medicine and Surgery
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Effects of Oral Fluids on the Dental Pulp

Previous Serial Number: None

Principal Investigator: Dr. T. Lundy

Other Investigators: Dr. H. R. Stanley

Cooperating Units: None

Man Years:

Total:	2-1/4
Professional:	1/4
Other:	2

Project Description:

Objectives:

1. To determine the effects of saliva on the dental pulp over a three-month period after a Class V cavity preparation was prepared and unrestored.

2. To establish a relationship between the clinical signs and symptoms and the microscopic pulpal changes.

3. To evaluate the rate of movement of bacteria and the depth of bacterial penetration in the dental tubules.

Methods Employed:

Class V cavities are prepared in human teeth, intact, restored, or slightly carious, and left open to saliva.

Although these specimens are slated for extraction for prosthetic or periodontal reasons, all are considered to be suitable for this study. None of the teeth show any clinical signs or symptoms prior to the procedure. Patients are accepted at all ages. The cavities

are prepared at the cemento-enamel junction or either the buccal or lingual intact surface with a Borden high-speed handpiece utilizing air-water spray and #2 or #4 bur. The preparation extends as deep into the dentin as possible and laterally to the mesial-distal borders. No preparations are left completely exposed to saliva without using any restorative material. Before the cavities are prepared and also at the time of extration, several diagnostic tests are performed to establish the level of response of sensitivity of the teeth, utilizing heat or cold stimuli, electrical pulp test, a dental probe tactile sensation test and a blast of air. Heat is applied utilizing gutta percha which is heated until it bends under its own weight. If a "no response" is obtained, a heated burnisher is then applied, because the burnisher maintains the heat for a longer period of time. If a "no response" is still obtained, then it is felt that the response is accurate and not a false negative. Cold is applied by two techniques: either utilizing a cotton pludget saturated with ethyl chloride or a cone of ice of standard size. In both the heat and cold tests various response times are recorded.

First, the time interval necessary for the patient to respond to either stimuli; second, the time interval necessary for the sensation to disappear. Stopwatches are used to record these intervals. Electrical pulp tests utilize the Burton vitalometer. The teeth are extracted under local anesthesia between one and 120 days postoperatively and routinely processed for microscopic interpretation.

Major Findings:

1. Heightened tooth sensitivity decreased as the time period increased and it was associated with an acute state of pulpal inflammation.

2. A delayed response to cold occurred in a group of teeth whose pulps had undergone the severest histopathological change.

3. Saliva can be detrimental to the pulp as evidenced by the formation of intrapulpal abscesses.

4. Bacterial penetration after 4 months averaged only 0.33 mm. from the cavity floor. Bacteria were found in the pulp only in the one specimen with a mechanical exposure.

Significance to Dental Research:

Previous studies by other investigators have been unsuccessful in attempting to correlate clinical signs and symptoms with pulpal pathology. None of the previous investigators had intentionally produced pulpal irritation and measured patient responses.

If a correlation could be established, the clinician's diagnostic ability would be greatly increased, and improved treatment offered. Knowing better the state of health of the pulp, the dentist could determine whether conservative or endodontic treatment would be recommended or necessary.

Few have investigated the effects of oral fluids on the dental pulp. Several investigators have mentioned that pulpal changes that have occurred under restorations are not due to the restorations themselves but are actually due to leakage at the interface. If saliva proves to be detrimental to the pulp, then the clinicians and dental material manufacturers would take an even greater effort in manufacturing and utilizing a material that more closely adapts to the tooth surface.

Proposed Course of the Project:

If a standarized pulpal response can be corrobrated into clinical signs and symptoms then various medicaments will be applied to counteract these changes. For instance, a corticosteriod preparation could be utilized to determine whether the steriod component can enhance healing of an irritated pulp, limit the extent of the reaction or have no effect.

Part B not included

Serial No. NIDR-91 (c) (67)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Histopathology of the Periodontal Ligament and Alveolar Bone Following Endodontic Treatment

Previous Serial Number: NIDR-71

Principal Investigator: Dr. T. Lundy

Other Investigators: Dr. H.R. Stanley

Cooperating Units: None

Man Years:

Total:	3/4
Professional:	3/4
Other:	0

Project Description:

Objectives:

To study the response of the periodontal ligament and alveolar bone following perforation of a previously intact root by an endodontic instrument or a bur.

Methods Employed:

Teeth scheduled for extraction for prosthetic reasons were used for this study. Routine endodontic treatment was performed utilizing the rubber dam in order to maintain aseptic conditions. The root canal was properly debrided, and prepared for obturation.

Before filling, a perforation through the previously intact root was made using an endodontic instrument or bur. Then the main canal was filled utilizing the laterally-condensed gutta percha technique. Therefore, only the area or the channel, which was created by the perforation procedure, was not filled. After various observation time periods the teeth were extracted with that portion of the involved labial bone left intact. The hard and soft tissues were fixed in

10 % formalin, and processed for microscopic evaluation. The specimen were serially sectioned in a horizontal plane. In this way, one could examine the response of the periodontal ligament, alveolar bone, and the dentin and cementum in the region of the perforation.

Major Findings:

The study is in process.

Significance to Dental Research:

Perforation of a previously intact root can occur from the following situations:

1. During endo-treatment, an endodontic instrument can penetrate the dentin and cementum of the root and involve the surrounding ligament and alveolar bone.

2. Perforation of the root can occur when placing a post into the root canal in order to construct a crown. Previously the perforation of a root surface would have resulted in the extraction of the tooth.

At present time there is a trend toward a more conservative approach. If a perforation ensues the dentist will flap the area, locate the perforation and seal it off with amalgam. Prognosis of these teeth subjected to an amalgam repair has not been determined, although it is considered to be quite favorable. Presently, the surgical approach is the only way of salvaging such teeth. There are several undesirable aspects to repairing the perforation surgically:

a. Post-operative discomfort.

b. The necessity of removing the labial plate of bone in the region of the perforation to gain access to the lesion for amalgam sealing. Unfortunately, in the removal of the alveolar bone, one might affect the stability of the tooth already involved by bone loss.

c. Unforeseen surgical problems may arise when, in throwing back a flap, one cannot always find the perforation, especially if it is located lingually or on a mesial or distal lingual aspect. In these cases some clinicians would extract the teeth while others will resort to periodic observations. This study will determine what events occur in the region adjacent to the perforation. Does the periodontal ligament and alveolar bone regenerate, or does the initial injury cause a subsequent degeneration and expansive destruction in

the area? If the study shows that the area can heal following a perforation then the clinician can resort to a non-surgical procedure in similar situations. The only procedure that the clinician would have to do is to fill the main canal, thereby not subjecting the patient to surgery.

Proposed Course of Project:

1. Even though the main canal is obturated, utilizing gutta percha with a root canal sealer, a small quantity of sealer will be extruded through the perforation into the injured alveolar bone and periodontal ligament. A thorough investigation of the toxicity of the root canal sealer on alveolar bone and periodontal ligament will be conducted in order to determine whether the cement impaired healing.

2. The extent of the initial injury to the supporting structures could have a decisive role in whether healing can be achieved. The size of the perforation will be correlated with the degree of healing.

Part B not included

376

Serial No. NIDR-92 (c) (57)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Histopathology of the Human Dental Pulp

Previous Serial Number: NIDR-72 (c)

Principal Investigator: Dr. H.R. Stanley

Other Investigators: Dr. H. Swerdlow, Dr. T. Lundy and Miss S. Knadle

Cooperating Units: School of Dentistry, Indiana University, Indianapolis, Indiana; U.S.P.H.S. Dental Health Center, San Francisco, California, Tufts College Dental School

Man Years:

Total:	2-1/4
Professional:	1/4
Other:	2

Project Description

Objectives:

1. To determine the range of pulpal responses to various operative and restorative procedures.

2. To determine the healing potential of normal and diseased pulp.

3. To determine the interval for odontoblastic regeneration and the rate of reparative dentin formation.

4. To determine the effects of steroid medication on the pulpal tissues and response.

5. To correlate clinical symptoms with microscopic pathology.

6. To determine the part saliva plays in intensifying the pulpal response.

7. To determine the mechanism of pulp stone formation.

8. To establish criteria for human pulp studies that will be

acceptable for forthcoming international standards.

9. To gain understanding concerning the cracked-tooth syndrome.

Methods Employed:

Patients are selected with non-carious, non-restored teeth to be extracted for prosthetic or periodontal reasons. Except for full crown procedures, most of the studies require Class V cavity preparations cut on the labial or buccal surface. The teeth are treated, restored and extracted at varying postoperative intervals to meet the requirements of a specific protocol. After fixation in formalin, the teeth are decalcified in 5% formic acid, embedded in paraffin, serially sectioned, and routinely stained with hematoxylin and eosin. Masson's trichrome, Wilder's reticulum stain, Feulgen's reaction, periodic acid-Schiff, toluidine blue, and other special stains are also utilized. Every attempt is made to standardize the categories to be compared in respect to age of patients, tooth size, postoperative extraction interval, and remaining dentin thickness. The pulpal reactions are compared by recording in incidence and intensity of the inflammatory response, cellular displacement and reparative dentin formation.

At present one collaborative research contract ($21,600) is underway at the University of Indiana School of Dentistry. Investigators there are evaluating the effectiveness of various types of liners beneath experimental epoxy resin restorative materials. At Tufts Dental College another collaborative research contract ($8,000) is nearing completion concerned with the effects of full crown procedures.

Major Findings:

1. When a prepared cavity was washed with a steroid formula containing 1% prednisolone in a vehicle of parachlorophenol, cresatin and gum camphor, before restoration with zinc oxide and eugenol, the average pulpal response was minimized from one-third to one-half. When the prednisolone was used without the vehicle, the inflammatory responses returned after 12 days. The long-term effect was sustained only in the presence of the vehicle. When the inflammatory responses resulting from cavity preparation were permitted to become fully established, the minimizing or modifying effect of the steroid formula was not observed until 48 hours after application. The steroid formula has definite efficacy for reversing an established focal pulpitis and shortening the resolution period.

2. The rather misleading statements in the literature concerning the rate of reparative dentin formation indicated a necessity for establishing some guidelines for this phenomenon. Little evidence of reparative dentin formation is apparent prior to the thirtieth

postoperative day. The rate of formation was highest initially in the 27-48 day interval (3.5μ /day); decreased markedly after the forty-eighth day of the experimental period to 0.7μ /day; and decreased further to 0.23μ in the 72-132 day period. One should not expect more than 0.2 mm of reparative dentin to be produced within 100 postoperative days.

3. Calcium hydroxide, when used as a cavity liner and not in direct contact with the pulpal tissues, did not increase the rate of reparative dentin formation. Only when actually controlling the pulp tissue was a stimulus apparent.

4. The condensation of gold foil even with the newer mechanical malleting instruments produces a considerable pulpal response when the gold foil is applied directly to freshly cut primary dentin not lined by reparative dentin. A Copalite lining plus a cement base will reduce the pulpal response by 50%. When gold foil is condensed against dentin lined by reparative dentin little or no pulpal response ensues.

5. Before we can determine the pulp responses of carious teeth to various experimental technics, we must determine how to predict the quantity of pulpal pathology to be found in the carious teeth prior to experimentation.

In a study based on the histological examination of non-operated carious human teeth, measurements were made between the most advanced point of bacterial penetration in the dentinal tubules and the pulpal tissues. As long as the cariogenic organisms were more than 1.0 mm from the pulp, the pulpal pathology was insignificant. When the bacteria reached within 0.75 mm of the pulp, a definite increase in pulpal pathology occurred. But not until the reparative dentin itself was invaded by the cariogenic organisms was significant pathology (abscess formations and chemically inflamed granulation tissue) found.

6. In the field of experimental epoxy resins, we have found them to be equal to silicate cements in terms of pulpal initiation. At this time they can be recommended for use only in conjunction with an impermeable liner. The collaborative research contract results are not yet complete. However, the liners presently being marketed with certain epoxy resins and considered acceptable by the profession are not providing adequate pulpal protection.

7. In a rat study utilizing tritiated thymidine, it was found that only one peak of mitotic activity occurred after operative injury. Ninety percent of the mitotic divisions occurred between 72 and 96 hours and differentiation of the daughter cells into odontoblasts was apparent.

Significance to Dental Research:

1. Contradictory findings in the dental literature leave the profession in a quandary concerning the use of certain operative technics and filling materials. Much of this confusion apparently has emanated from the lack of appreciation of various investigators for the response of the pulp to operative trauma per se. Pulpal response can vary in incidence and intensity according to the speed and pressure employed in instrumentation, the thickness of remaining dentin, the postoperative extraction period, the type of coolant, the size of the cutting tool, the amount of salivery contamination. All of these factors must be considered before evaluating accurately the additional irritating properties of permanent and temporary filling materials, cavity liners and sterilizing agents.

2. Specific biologic problems related to dental procedures are occuring at an ever-increasing rate and require a stepped-up program as described in this report to keep pace with the needs of the dental profession.

Proposed Course of Project:

1. With the high speed technics, the traumatic effect of cutting tooth structure is minimal enough that the incidence of reparative dentin is greatly reduced. Consequently, virgin tubules opened by operative technics remain open and permit the filtration of toxic products of cement and silicate to reach the pulp tissues. Measures must be found to either increase the incidence of reparative dentin formation and/or seal these tubules adequately against permeating substances.

2. There is a need for describing the significant events within the entire panorama of odontoblastic regeneration. Although much of this information can be derived from fixed material, studies are in progress utilizing tritiated thymidine on rats and primates.

3. In order to cope with the increasing number of biologic problems in this field contract arrangements will be continued with various dental schools and hospitals to secure additional specimens for our studies.

Part B

Honors and Awards:

1. Elected to President of the American Academy of Oral Pathology, April 5, 1967.

2. Formal consultant to U.S. Naval Dental School, National Naval Medical Center, Bethesda, Maryland.

3. Appointed to Subcommittee of F.D.I. on Toxicity of Filling Materials.

Publications:

1. Stanley, H.R., Swerdlow, H. and Buonocore, M.G.: Human pulpal response to experimental restorative resins. J.A.D. A.: 75:132-141, July , 1967.

2. Stanley, H.R.: Design for a human pulp study. Oral Surg., Oral Med., and Oral Pth.

3. Stanley, H.R. and Weaver, K.: A technique for the preparation of human pulpal tissues. Accepted for publication in a workshop monograph entitled "Biology of the Dental Pulp Organ."

4. Sundell, J.R., Stanley, H.R.: The relationship of coronal pulp stone formation to experimental operative procedures, Oral Surg., Oral Med., and Oral Path.: April, 1968.

5. Stanley, H.R. Dental Science Handbook, Chapter on "Oral Pathology". To be published by the American Dental Association.

6. Stanley, H.R.: Human pulpal response to new drugs and restorative materials. Mass. Dental Society Journal 16: 80-84, Spring, 1967.

7. Stanley, H.R.: "The cracked tooth syndrome". To be published in the Journal of the American Academy of Gold Foil Operators.

Serial No. NIDR-93 (c) (66)
1. Oral Medicine and Surgery
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Autogenous Replantation of Human Teeth

Previous Serial Number: NIDR-73 (c)

Principal Investigator: Dr. H. R. Stanley

Other Investigators: Drs. P. N. Baer, P. Lightbody, H. Swerdlow and T. Lundy

Cooperating Units: None

Man Years:

Total:	2-1/4
Professional:	1/4
Other:	2

Project Description:

Objectives:

1. To observe in time sequence the reaction of the periodontal tissues to autogenous reimplantation of human teeth.

2. To establish the regenerative period for cementoblasts.

3. To study the phenomena of ankylosis and resorption.

4. To determine why a cemental surface that has been involved by a periodontal lesion is apparently incapable of offering a suitable surface for the deposition of new cementum.

Methods Employed:

On specially selected patients that have teeth to be extracted for prosthetic or periodontal reasons and who will eventually receive full dentures, one or two teeth were extracted, received endodontic therapy, cleansed and re-inserted into the same tooth socket. Before re-insertion, portions of the cemental surface were scored with a rotary cutting instrument to expose the primary dentin. Each re-implanted tooth was splinted until reattachment and stability occur-

red. At intervals of two weeks, one month, three months, and six months, the teeth were again removed with their attached tissues. The specimens were properly fixed and processed for microscopic evaluation. The specimens were serialed horizontally from the occlusal or incisal surface to the apex. Particular attention was given to the status of the cementum, whether that cementum initially left had resorbed or been coated with new cementum and whether new cementum had been deposited on the exposed dentin. Characteristics of immune rejection represented by resorption and ankylosis were detailed.

Major Findings:

1. Most of the reimplanted teeth are accepted and become firmly attached. A few teeth are exfoliated within several days. This is a problem in itself that needs clarification.

2. New functioning cementoblasts have not been found earlier than 29 days. Incidentally the regeneration of odontoblasts with production of reparative dentin takes 30-35 days.

It is possible that the same interval of time is required for the differentiation of cementoblasts.

3. It appears that resorption is generally accompanied by ankylosis. It must be determined whether resorption will occur in the complete absence of ankylosis.

Significance to Dental Research:

Through the centuries tooth replantation has been attempted. Present-day methods are not much more successful than experienced 100 years ago. However, with progress in the science of immunology, the reasons for failure are now more understandable. Replanted teeth do not produce a typical rejection phenomenon but are eventually resorbed and exfoliated after about 18 to 24 months. To date no one has carried out a basic study of the initial and progressive tissue responses related to human replanted teeth in time sequence fashion. Instead, the teeth are usually permitted to remain in the mouth until exfoliated and then examined by a pathologist.

For a long time, the periodontists have been stating that their best clinical results in regard to reattachment of the periodontal tissues occurred within an intrabony pocket. Reattachment otherwise has been generally unsuccessful, the clinician being satisfied to obtain shrinkage of gingival tissues with increased tissue tone. Recently,

however, long term clinical studies have shown, unfortunately, that intrabony pockets offer no particular advantage for reattachment.

In other words, a cemental surface that has been involved by a periodontal lesion is apparently incapable of offering a suitable surface for the deposition of new cementum, a requirement for reattachment of periodontal collagen fibers.

In other words, the problem of reattachment facing thousands of periodontists throughout the world, is the same problem thwarting the success of autogenous replantation of human teeth. Any knowledge that might be derived from the study of the autogenous replantation of human teeth could also be beneficial to the entire practice of periodontology.

Proposed Course of Project:

1. During the first 18 months of this program, nineteen patients have been included in the study. During the coming three-year period we hope to obtain a total of fifty cases.

2. There is a new industrial technique for coating glass which makes it more acceptable for tissue culture growth. Our interest in this technique is to determine whether a thin coating of this same material on a duplicated acrylic tooth before replantation in the original socket would provide a surface suitable for the differentiation of cementoblasts and the deposition of cementum.

3. Some recent studies have indicated that decalcified bone encourages bone regeneration at an amazing rate. It is planned to subject the root surface of teeth to minimal decalcification in order to affect the outer surface but not enough to weaken the total structure of the tooth.

Part B not included.

Serial No. NIDR-94 (c) (44)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Studies on the Etiology and Control of Rampant Dental Caries: Clinical and Experimental Animal Studies on the Differentiation of Cariogenic and Non-cariogenic Foods

Previous Serial Number: NIDR-74 (c) (44)

Principle Investigators: Dr. R. M. Stephan

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives:

Rampant dental caries is a very severe form of the disease in which practically all of the teeth are attacked by decay in a relatively short period of time. It is found chiefly in young children, but may develop in adults who previously had little or no caries experience. Under suitable experimental conditions, comparable forms of rampant caries can be developed in laboratory animals such as rats and hamsters. From a research standpoint, rampant caries offers a most favorable opportunity to study the basic factors which activate or control the caries process because the usually prolonged time element in the development of carious lesions is reduced to a minimum, and the determination of caries activity can be much more certain than in caries of usual severity. The purpose of this project is to evaluate in clinical studies the many factors which may be important in different cases of rampant caries, and to study the more important of these factors in laboratory and animal experiments with the goal of establishing more effective means for solution of the caries problem.

Methods Employed:

1. Clinical Studies

In addition to the usual oral and medical examination and laboratory tests, a detailed history is taken in regard to: (a) caries experience in the family; (b) diet and drinking water, illnesses and medication during the period of tooth formation; (c) food selection, eating habits and other factors affecting oral hygiene and food retention around the teeth during the period in which caries developed; and (d) a comparison with these factors in siblings and other patients with minimal caries.

Direct stereomicroscopic observations are made on the growth of bacterial plaques and the retention of food material in carious tooth surfaces as compared with intact tooth surfaces, using the Zeiss "otoscope" at 6X to 40X magnification. pH measurements are also made in these areas and salivary flow rates are measured.

A large number of plaque samples for the fluorescent antibody study of specific strains of alpha streptococci which had accumulated from previous years, have now been studied by Mrs. Shirley Geis and this part of the rampant caries study is reported by her in a separate project report.

2. Laboratory Animal Experiments

The clinical studies have indicated that the frequent eating of foods containing sucrose or other fermentable carbohydrates is a very important factor in the etiology of rampant caries. During the past year animal experiments using OM and pathogen free SD rats have been continued to find foods which may not be cariogenic even though they contain considerable amounts of fermentable carbohydrates. These experiments have included the addition of graded levels of sucrose or glucose to some of the world's basic foods which are relatively non-cariogenic, such as milk, whole wheat, meat and fish meal, or fish protein concentrates. The sucrose or glucose was given in several ways, either mixed with the food tested, given separately in a second food cup, or given as a solution in the drinking water. In addition certain minerals, including calcium salts, phosphates, calcium phosphate, magnesium, sodium or calcium fluoride were added at graded levels to the sucrose or glucose to determine the relative extent to which these additions would inhibit caries.

Major Findings:

The study of new patients with rampant caries during the past year has further extended our knowledge of the conditions under which

rampant caries develop. The social and economic situations which lead people to substitute frequent between meal eating for regular meals was again emphasized. For example, a child had developed rampant caries six months after his mother had started working and was no longer at home during the day to feed him. An adult patient developed rampant caries within a year after she had started sucking candy mints to replace her previous cigarette smoking habit. The pattern of eating sweets between meals has been a common finding in rampant caries patients.

Previous animal experiments have shown that some foods commonly eaten by people, such as sucrose, glucose, candies, cookies, some fruits, bread with jelly and similar foods containing fermentable carbohydrates were highly cariogenic to rats, whereas other foods such as milk, whole wheat, peanuts, popcorn, cheese, and similar materials were relatively non-cariogenic to rats.

During this past year we have continued our rat experiments on the effect of fish protein concentrate (F.P.C.) and fish meals to reduce the cariogenic effects of sugar and other cariogenic foods when mixed with them; although F.P.C. contains from 100 to 250 ppm. fluoride, this fluoride probably accounts for only part of the anticariogenic effect since in other experiments in which comparable amounts of fluoride were added to diets containing 66% or 83% of sucrose, the inhibition of caries was not as great as with the fish products.

Some of the anticariogenic effect is probably due to the relatively high level of calcium, phosphorus and basic amino acids in F.P.C., as well as to its effect in raising the nutritional value of high carbohydrate foods such as sugar and cereals.

Significance to Dental Research:

The chief significance of the clinical studies is to point up the problem of between meal eating of sweets and snacks as conducive to the high rate of dental caries in some members of one population, and to indicate the need for both more regular eating habits, and the selection of non-cariogenic foods for between meal eating. In addition, the need to develop more non-cariogenic snacks and to make them available for people instead of the usual cariogenic sweets is obvious. In this regard the demonstration that fish protein concentrates exert a great anticariogenic effect on sucrose when fed to laboratory rats suggests that this material and food supplements like it may not only be helpful in overcoming human malnutrition, but also in the control of caries.

Proposed Course of Project:

It is planned to follow up the findings evaluating the cariogenic and anticariogenic properties of different food materials, particularly the effects of fish protein concentrates, and to complete analysis of the extensive clinical data which have been developed on rampant dental caries and its control.

Part B not included

Serial No. NIDR-95 (c) (66)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Studies of Oral and Pharyngeal Form and Function in Infants

Previous Serial Number: NIDR-76 (c)

Principal Investigator: Dr. J. F. Bosma

Other Investigators: None

Cooperating Units: Dr. W. J. Logan, Stanford University; Dr. M. Silbiger, Division of Radiology, Clinical Center, NIH; Division of Photography, NIH

Man Years:

Total:	1/4
Professional:	0
Other:	1/4

Project Description:

Objectives:

To describe development of functions of pharynx and mouth in the normal human infant and in infants impaired by malformation or by neurological disorder.

Methods Employed:

Cineradiographic, cinephotographic and acoustical methods of observation of suckle feeding and of respiratory functions, including cry.

Major Findings:

Studies have been obtained on 8 additional impaired infants including two examples of pharyngeal incoordination of the newborn, and one of familial dysautonomia.

A form of swallow recognized in the mature human as "pint swallow", of sustained opening of the pharynogoesophageal segment without elevation of the hyoid and larynx, was found for the first time in an infant.

The classification of mechanisms of infant pharyngeal disabilities, described in 1967 by Logan and Bosma, has been extended to include criteria of potential mobility of the pharynx, which may be limited in its displacements by muscle contractures.

Significance to Dental Research:

The disabilities of function and form in childhood, which are the usual concern of dentists, originate in earlier years. Definition of disabilities in infancy, and description of their evolution into the patterns of disordered function in childhood, should provide meaningful etiologic perspective to the dentistry of impaired children.

Proposed Course of Project:

Continuation of these studies.

These observations are being accumulated into a portion of a general publication: Development of Form and Function of the Normal and Abnormal Pharynx.

Part B not included

390

Serial No. NIDR-96 (c) (66)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Studies of Development of the Head Skeleton of the Rat

Previous Serial Number: NIDR-75 (c)

Principal Investigator: Dr. J. F. Bosma

Other Investigators: None

Cooperating Units: University of Michigan, School of Dentistry Dr. M. Baer and Miss E. Hirshfeld; Dr. J. Ackerman, National Library of Medicine - by a Special Publication Grant; Colorfax Laboratories, Inc.

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives:

Demonstration of patterns of growth of the head skeleton of the rat, as a representative mammal.

Methods Employed:

Rats were alizarinated in different groups at ages selected to illustrate specific phenomena of development of individual head bones.

The sites of successive red and blue deposition are demonstrated by thin sectioning of rat skulls. Unstained individual bones were also dissected at 15, 33, and 130 days and photographed in standard comparison orientations. A notable element of this project is the form of its publication in a major volume, An Atlas of the Postnatal Development of the Rat Skull, with atlas-style illustrations and 120 matching color transparencies. The preparation and duplication of these slides for each of 1500 copies of the book is sponsored by

the National Library of Medicine as a publication demonstration project. In collaboration with Dr. Baer and Miss Hirshfeld, Colorfax Laboratories has developed special printing procedures for this project.

Major Findings:

By these methods, it has been possible to demonstrate the patterns of incremental growth and modulation of form of individual bones, both separately and in situ, as well as the resultant increments and form modulations of general areas of the skull.

Significance to Dental Research:

This study demonstrates and elucidates the patterns and principles of skull growth in a mammal. These patterns and principles are applicable to mammalia, in general.

These particular demonstrations, in the rat, afford baselines for evaluation of experimental deformations in the laboratory rat.

Proposed Course of Project:

The book, An Atlas of the Postnatal Development of the Rat Skull, under authorship of M. Baer, J. Bosma, and J. Ackerman, is scheduled for submission to the Government Printing Office in FY 1969. The projection transparencies and matching drawings will be completed in May, 1968.

In this continuing project, Dr. Baer has alizarinated at selected ages a basic series of 24 pigs, as representatives of omnivora, and 14 sheep, as representatives of herbivora. These animals were grown and sacrificed and the skulls are now ready for sectioning. The study will also be extended to rabbits.

Part B

Publications:

1. Baer, M. J., and Ackerman, J. L.: "A Longitudinal Vital Staining Method for the Study of Apposition in Bone" in Studies on the Anatomy and Function of Bone and Joints, Springer-Verlag, Berlin, 1966.

As an extension of this project interest, Dr. M. Baer has also arranged and conducted a Symposium on In Vivo Bone Markers, presented at the 1967 Meeting of the American Association of Physical Anthropologists. The contents of this Symposium will appear as a single issue (July, 1968) of the American Journal of Physical Anthropology.

Serial No. NIDR-97 (c) (62)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Studies of Sensory and Motor Functions in Subjects Impaired by Malformations of Neurological Disease

Previous Serial Number: NIDR-77 (c) (62)

Principal Investigator: Dr. J. F. Bosma

Other Investigators: Dr. R. D. Christensen, Dr. R. L. Christiansen, Dr. L. Krames, and Dr. B. Weinberg

Cooperating Units: Dr. D. Brodie, National Institute of General Medical Sciences; Dr. R. Henkin, National Heart Institute; Dr. J. Kavanagh, National Institute of Child Health and Human Development; Dr. M. Silbiger, Clinical Center Division of Radiology and Division of Medical Illustrations and Photography; Cleft Palate Clinic of the Johns Hopkins Medical Center; University of Indiana Department of Neurology; Department of Speech, Catholic University of America.

Man Years:

Total:	1 1/2
Professional:	1/2
Other:	1

Project Description:

Objectives:

Continuation of studies of sensory, perceptual and motor mechanisms in the mouth and pharynx.

Methods Employed:

The clientele continue to include a variety of subjects impaired by malformation and/or neurological impairment in the oral and pharyngeal area. The basic study methods continue to include standard routines of cinephotography, cine and still radiography, etc.

Major Findings:

The correlative studies of anatomical form and of sensory and motor function of an array of persons having primary abnormalities of function has afforded opportunities of recognition of abnormalities of form in the neurologically impaired and, reciprocally, of abnormalities of function in those malformed by antenatal anomaly.

Particular studies have been performed in 3 subjects having severe hypoplasia of the tongue. Clearly articulated speech is achieved in two of these by distinctive motions of the lips, and in all three by distinctive motions of the dorsal portion of the anomalous tongue mass in relation to the soft palate and the walls of the mesopharynx. Our appreciation of the potential mechanisms of speech articulations are thus increased. Following identification of articulatory valving in the lateral portion of the lip, this lip manuever was discovered in corresponding /s/ articulation in an adult who had learned new speech articulation after surgical excision of a major part of his tongue, in therapy of cancer.

This correlative study approach has also been applied to children having hypoplasia of the facial skeleton and submucous cleft palate. In 5 children having anatomically similar patterns of hypoplasia and similar abnormalities of speech and of feeding, R. Henkin found hypesthesia of the palate and similarly patterned abnormalities of taste and smell; their form of hyposmia (designated "type 2") was found also in their mothers. Analogous coincidences of abnormalities of facial form, of smell and of taste were found in subjects with Turner's syndrome of hypogonadism.

Since different motor mechanisms are employed by the oral and pharyngeal area in feeding and in speech functions, these two categories of function were observed in comparison for the demonstration of potentialities of motor performance. This comparison has been made in the upper pharyngeal function of cleft palate children. In most children with hypoplasia of the palate and in some with simple cleft palate who have had the usual form of surgical repair of the cleft, the upper pharynx closes adequately in the initial phase of swallow; nasal regurgitation of bolus does not occur. But the palatopharyngeal isthmus fails to close consistently or adequately in speech, and these subjects are correspondingly "hypernasal." This duality of performance mechanisms is also applicable to the analysis or study of neurological impairments. In the current report year, studies were brought to publication in two neurological syndromes which are manifested principally in impairments of the motor unit; amyotrophic lateral sclerosis and myotonic dystrophy. In each of these conditions, discrepancies were found between the actions of the mouth and pharynx in feeding actions, which were commonly adequate in function,

compared with the actions of speech and the respiratory function of maintenance of the pharyngeal airway, which were relatively more distorted and impaired. This approach of dual observation of separate motor functions is applicable to other forms of neurological abnormality. A similar discrepancy between adequacy of feeding functions and failure of speech functions was reported previously in two clinically similar subjects whose syndrome of abnormality was defined by deficiencies and impairments of oral sensation and perception (Bosma, J.F., Grossman, R. C., and Kavanagh, J. F., A Syndrome of Impairment of Oral Perception, Chapter 18, in First Symposium on Oral Sensation and Perception, 1967).

The tests of oral form perception described in the first Symposium on Oral Sensation and Perception were further standardized by Dr. Weinberg, assisted by a graduate speech student from George Washington University and another student from Catholic University of America. Time parameters of the test were employed, and were found to afford separate additional criteria of test performance. A major effort has been devoted to the arrangement of a Second Symposium on Oral Sensation and Perception, and its transcription, editing and preparation for a volume publication. The contributions included 11 NIH Intramural and 18 Extramural persons. The publication is of 29 titled presentations, of 180 edited discussion items and 4 final comments. Its estimated text is 660-680 pages. Included are original publications on mechanisms of taste sensation, on central representations of oral area afferents, on reflex functions of the mouth of the fetus and the neonate, on oral sensation and perception testing in normals and in subjects impaired by neurological disease.

Significance to Dental Research:

The increments of understanding of oral and pharyngeal function in normal and in impaired subjects are strategic to the development of additional techniques of study and therapy and of extension of dental therapy to additional categories of impaired persons.

Proposed Course of Project:

Continuation of current study routines, graduating generally to clientele of neurologically impaired subjects, and fewer subjects impaired by primary malformation in the facial area.

395

Part B

Publications:

1. Henkin, R.: Impairment of Oral Sensation and Perception and Hyposmia in Association with Facial Hypoplasia and Growth Retardation, Chapter in publication in Second Symposium on Oral Sensation and Perception, in preparation.

2. Weinberg, B., Bosma, J. F., Shanks, J. C., and DeMyer, W.: Myotonic Dystrophy Initially Manifested by Speech Disability, J. of Speech and Hearing Diseases, 33:51-58, 1968.

3. Bosma, J. F. and Brodie, D.: Disabilities of the Pharynx in Amyotrophic Lateral Sclerosis, as Demonstrated by Cineradiography, submitted to Neurology.

4. Bosma, J. F.: Editor, Second Symposium on Oral Sensation and Perception, in preparation.

5. Weinberg, B., Lyons, M. J., and Liss, G. M.: Studies of Oral, Manual, and Visual Form Identification Skills in Children and Adults, in Second Symposium on Oral Sensation and Perception, in preparation.

6. Weinberg, B., Liss, G. M. and Hillis, J.: A Comparative Study of Visual, Manual, and Oral Form Identification in Speech Impaired and Normal Speaking Children, in Second Symposium on Oral Sensation and Perception, in preparation.

7. Henkin, R. I., Christiansen, R. L., and Bosma, J. F.: Impairment of Oral Sensation and Perception and Hyposmia in Association with Facial Hypoplasia and Growth Retardation, in Second Symposium on Oral Sensation and Perception, in preparation.

8. Henkin, R. I.: Manual and Oral Stereognosis in Normal Volunteers and in Patients with Various Abnormalities of Taste and Olfaction, in Second Symposium on Oral Sensation and Perception, in preparation.

9. Henkin, R. I.: The Neuro-Endocrine Control of Sensation and Perception, in Second Symposium on Oral Sensation and Perception, in preparation.

Serial No. NIDR-98 (c) (66)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Serial Extraction Study on Preadolescent Children Having Crowded Class I Occlusion

Previous Serial Number: NIDR-51

Principal Investigator: Dr. R. D. Christensen

Other Investigators: Dr. L. F. Mills, and Dr. R. L. Christiansen

Cooperating Units: Carole Highland Elementary School; Holly Park Elementary School; O. W. Phair Elementary School; and Hollywood Elementary School, Prince Georges County, Maryland

Man Years:

Total:	1 1/4
Professional:	3/4
Other:	1/2

Project Description:

Objectives:

1. Compare the changes which occur within the dentition of subjects who undergo serial extraction, and subjects with a similar diagnosis but do not receive extractions. Special notice will be made of arch width, length, crowding of teeth, overjet, overbite, root length and formation, and status of supporting tissues.

2. Compare the degree and direction of development of the facial bones of these two groups. Special interest will be taken in observing the size of maxilla and mandible achieved within these two groups.

Methods Employed:

Intraoral mirror examinations on approximately 1200 first, second, and third grade children in four elementary schools have been performed. Initial identification of sample subjects was based on clinical evidence of:

1. moderate to severe crowding of permanent anterior teeth.
2. harmonious size, form, and relationship of maxilla and mandible
3. good oral hygiene and minimal decay of teeth
4. a mixture of deciduous and permanent teeth present in the mouth
5. medically healthy and normal and oral and facial muscle function

Subjects found to meet these criteria will undergo more through examination including collection and analysis of the following records: (a) medical and dental histories; (b) detailed mouth examination; (c) five cephalometric radiographs; (d) radiographs of individual teeth; (e) orthodontic study models; (f) facial and intraoral photographs; and (g) medical examination.

Those subjects found after thorough examination to meet the most rigid criteria for serial extraction procedures will be re-examined every six months until the permanent dentition is complete.

Major Findings:

The preliminary examinations of 1200 children have revealed:

1. a wide range of variation in permanent tooth eruption time and sequence
2. an apparently closer correlation between time of tooth eruption and other physical growth parameters than exists between eruption time and chronologic age
3. a lower incidence of subjects meeting the criteria for the long term study than was anticipated.

Significance to Dental Research:

Serial extraction procedures are used frequently in dentistry to help guide the eruption of permanent teeth into more favorable positions in the dental arches. The procedure was designed to anticipate and hopefully prevent the development of a fully matured deformity in the permanent dentition. It is applied by extraction, in proper sequence, of a predetermined series of deciduous and permanent teeth. Such procedures are presented in graduate and undergraduate orthodontic texts as a method of interceptive

orthodontics, following a thorough diagnosis, which will not necessarily result in an ideal occlusion but will produce a more desirable alignment of teeth. There are, however, questions about serial extraction which still remain unanswered including documentation of the concomitant changes occurring dentally and skeletally and their frequency of occurrence.

Proposed Course of Project:

The findings of the preliminary screening examinations have suggested a need for revision of subject selection criteria before continuing the long term study proposed. The findings related to variation in tooth eruption are being more thoroughly analyzed in anticipation of publication.

Part B not included.

Serial No. NIDR-99 (c) (67)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Comparative Skeletal and Soft Tissue Cephalometric Analysis of Acromegalic and Normal Human Adults

Previous Serial Number: None

Principal Investigator: Dr. R. D. Christensen

Other Investigator: None

Cooperating Units: Dr. J. Roth and Dr. P. Gorden, Clinical Endocrinology Branch, National Institute of Arthritis and Metabolic Diseases

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

1. Describe the skeletal and soft-tissue changes in the cranio-facial complex of individuals with acromegaly.

2. Assess changes, if any, in cranio-facial skeletal and soft tissue features of the acromegalic following irradiation of the pituitary tumor.

Methods Employed:

Lateral cephalometric headfilms on 27 male and 15 female humans with acromegaly have been analyzed. Similar measurements have been made on headfilms of 20 male and 20 female humans with no evidence of acromegaly. Comparative statistical analyses have been employed to determine areas of significant difference between the acromegalic and non-acromegalic samples.

Additional headfilms have been taken at annual intervals following irradiation of the pituitary tumors of approximately 20 of the acromegalic patients. These are analyzed and compared with the pre-irradiation analyses.

Major Findings:

1. The greatest differences between groups was (a) increased thickness of soft tissue at the midsagittal plane in the region of lower lip and chin in the acromegalics, (b) increased mandibular length, thickness, and density in the acromegalics, (c) increased area (measured with a planimeter) between mid-sagittal soft tissue profile and a plane from nasion through pogonion in the acromegalics.

2. No significant change in skeletal or soft tissue measures in the acromegalics treated by irradiation.

3. Greater soft tissue profile thickness in the acromegalics than in a group of 13 young adults with prognathic mandibles but no evidence of elevated growth hormone levels.

Significance to Dental Research:

Many questions regarding mechanisms of growth of the facial complex are unanswered. The role of growth hormone in normal facial growth is not understood. The description of facial changes in the presence of elevated growth hormone provides information necessary for a better understanding of facial growth processes.

Proposed Course of Project:

The patients who have had radiation therapy should continue to have periodic radiographic evaluations. All new patients with acromegaly entering the Clinical Center should be included in this study.

Part B not included.

401

Serial No. NIDR-100 (c) (66)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Study of Taste Thresholds, Tastebud Distribution, and Associated Dentofacial Form

Previous Serial Number: NIDR-78 (c)

Principal Investigator: Dr. R. L. Christiansen

Other Investigator: Dr. J. F. Bosma

Cooperating Units: Dr. R. I. Henkin, National Heart Institute

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description:

Objectives:

To define the specific area or areas of the oral cavity responsible for normal detection and recognition of the four basic taste modalities in normal subjects and in edentulous subjects habitually wearing dentures.

Methods Employed:

Data on baseline taste and smell thresholds and associated oral and facial forms have been secured on ten young normal subjects over 21 years of age. The taste and smell examinations involved forced choice among three solutions. Taste is tested under four conditions: no oral anesthesia, palatal anesthesia only, lingual anesthesia only, and both palatal and lingual anesthesia. Infiltration of Lidocaine is performed to produce anesthesia of the hard and soft palate and the tongue.

Major Findings:

The taste receptors on the palate are concentrated at the midline near the junction of hard and soft palate. Reception on the tongue is primarily at the tip, lateral borders, and occasionally on the dorsum, near the tongue base. When one surface is anesthetized the physical contact of tongue against palate still greatly facilitates taste perception regardless of which surface has been injected. Under anesthesia of the palate and tongue, little oral taste perception persists. In this condition it is possible to determine thresholds of taste in the pharynx after swallowing the test solution.

Significance to Dental Research:

Recent observations by Drs. Bosma, Henkin, and Christiansen on five children with deficiencies of taste and olfaction, and orofacial development suggest a possible relationship between the development of these special senses and malocclusions resulting from skeletal discrepancies.

Proposed Course of Project:

Continue study of the senses of taste and smell in normal subjects, followed by studies on patients with specific forms of malocclusion.

Part B

Publications:

1. Henkin, R. I. and Christiansen, R. L.: Taste Localization on Tongue, Palate and Pharynx of Normal Man, J. Appl. Physiol. February, 1967.

2. Henkin, R. I. and Christiansen, R. L.: Taste Thresholds in Patients with Artificial Dentures, J. Am. Dent. Assoc. 75:118, 1967.

Serial No. NIDR-101 (c) (65)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Study of Oral Area Motor Mechanisms by Use of Pressure Transducers

Previous Serial Number: NIDR-79 (c)

Principal Investigator: Dr. R. L. Christiansen

Other Investigators: None

Cooperating Units: Instrument Fabrication Division, NIH; Dr. W. R. Proffit, University of Kentucky, Department of Orthodontics; Dr. R. E. McGlone, Department of Speech, State University of New York, Buffalo

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description:

Objectives:

1. To further improve performance and reliability of intra-oral pressure transducer designs.

2. To obtain data on pressure-time integral for forces acting on the teeth.

3. To obtain data regarding tongue positioning and activity during swallowing and articulation of selected consonant sounds.

Methods Employed:

In cooperation with NIH Instrument Fabrication Division, two types of pressure transducers have been constructed, dynamically calibrated and tested by this section. The cantilever-beam transducers utilize resistance strain gauges and the miniature transducers use foil or semiconductor strain gauges.

Tongue activity during speech and swallowing was studied in ten normal control subjects. The test instrument consisted of a removable maxillary appliance containing three transducers, two mounted bilaterally, lingual to the first molars, and one located just palatal to the central incisors.

Planimetric determination of the area under the pressure curves, the pressure-time integral, was used in data collection. Computerization of pressure-time integral measurement is being explored.

Major Findings:

It appears that 10 cps is the maximum fundamental frequency of lingual pressure waves even during rapid speech. Dynamic calibrations revealed that the cantilever-beam transducer has frequency response characteristics linear beyond 150 cps while the diaphragm transducer has linear response to 25-30 cps. Therefore, both designs give an adequate safety factor for studies of tongue movements and pressures. The diaphragm design offers the improvements of reduced size with increased output characteristics.

Lingual pressures were measured during speech of the ten normal control subjects for various consonant-vowel combinations (/di/, /id/, /ta/, /at/, /da/, /ad/, etc.). Results indicated that mean lingual pressures for the consonants were unaffected by changing the vowel within the syllable. However, differences in pressure values were found between each of the consonants when syllabic position and vowel were held constant. Pressure values for the consonant in the initial position were greater than the mean values for the same consonant in the final position. A further difference was found in the time relation of maximum pressure to the onsent of phonation for each of the consonants. Greater pressure values were obtained from rapidly produced syllables as compared with slow speech utterances.

Results showed considerably greater integrated pressure associated with swallow pressure values than with any speech activity. The female subjects exhibited greater lingual pressure during swallowing than did the males. This relationship was not found during either speech activity involving rapid and normal rates.

Signifieance to Dental Research:

Muscle pressures play an ill-defined but probably important role in the normal development of the dentition and in the etiology of malocclusion. This study is designed to better define that role.

405

Proposed Course of Project:

Continued instrumentation development and intensive analysis of intra-oral pressure patterns during speech and swallowing activity.

Part B

Publications:

McGlone, R. E., Proffit, W. R., and Christiansen, R. L.: Lingual pressures associated with alveolar consonants, J. Speech and Hearing Research, 10:606, 1967.

Proffit, W. R., Fogle, J. L., Heitlinger, L. W., Christiansen, R.L., and McGlone, R. E.: Dynamic calibration of lingual pressure transducers, J. Applied Physiology, 21:1417, 1966.

406

Serial No. NIDR-102 (c) (68)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Restitution of Mandibular Form After Condylar Injury

Previous Serial Number: None

Principal Investigator: Dr. P. J. Coccaro

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	2/3
Professional:	2/3
Other:	0

Project Description:

Objectives:

1. To prove that facial asymmetry (due to trauma of mandibular condyle at age 2) becomes progressively more severe because of accompanying aberrant muscle function on the affected side.

2. To demonstrate influence of stimulating muscular activity thus creating favorable growth changes in condyle, ramus and body of hypoplastic hemi mandible in a child with facial asymmetry.

3. Demonstrate annually with photographs and radiographs a diminution of facial asymmetry supported by favorable skeletal changes in condylar and mandibular growth on the side of mandible which previously exhibited growth arrest.

Methods Employed:

An occlusal index was made in acrylic with patient deviating as far laterally to the unaffected side. The appliance was cemented over deciduous teeth and patient wore this and similar appliances for 3 years. The rationale behind such therapy was clinical evidence that the patient had marked limitation of lateral and protrusive mandibular movements originating from the affected side. The appliance

compelled the child to more effectively utilize muscles (internal and external pterygoids) on the affected side.

Major Findings:

1. Clinical and radiographic evidence over a 7 year period demonstrate a dramatic diminution of facial asymmetry present when patient first came to clinic.

2. Initial laminographs of temporo-mandibular joint show marked hypoplasia of condyle, shallow glenoid fossa, short ramus and body as well as ante-gonial notching, all present on the affected side of patient's mandible before treatment. Final laminographs of temporo-mandibular joint exhibited the impact of muscular activity through the use of an occlusal guide plane. Restitution of condylar form and a glenoid fossa that became less shallow and more concave. Ramus height and body length reflect demonstrable growth over the years of study.

3. Opening, closing and protrusive motions are more in line with normal parameters since being treated. This plus improved facial appearance is in direct proportion to obvious skeletal changes noted on the affected side.

4. Study indicates that abnormal function could very well tend to compound severity of facial asymmetry, after initial trauma, and if an effort is made early enough progressive facial asymmetry could be reduced or eliminated.

5. P. A. radiographs, over a seven year period, show the change in facial appearance (due to favorable growth which created an equalization of structure size on both sides that was non-existent before.

Significance to Dental Research:

Traumatic injuries to the mandible in preschool children can produce damage to the growth site in the mandibular condyle. Facial asymmetry usually ensues and reports in the literature have indicated that it becomes progressively worse with growth and development. Abnormal mandibular movements in opening and closing and protrusion have also been noted in these children. Such patients are usually relegated to "waiting and watching" periods and recommendations for surgery when they have reached maturity. Other have had many surgical procedures to correct the size disparity on one side with the other.

This study certainly indicates that improved muscular functional activity, when restored early, can contribute significantly to a diminution of facial asymmetry resulting from favorable growth on affected side.

Proposed Course of Project:

More cases of this type should be followed and results documented to further substantiate the impact of muscular activity in reducing or minimizing the progressive facial asymmetry which is expected with growth and development.

Part B not included

Serial No. NIDR-103 (c) (68)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Clinical and Roentgenographic Analysis of Orthodontics (on a Continuing Basis) in Cleft Palate Habilitation

Previous Serial Number: None

Principal Investigator: Dr. P. J. Coccaro

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	7/12
Professional:	1/3
Other:	1/4

Project Description:

Objectives:

1. To study the impact of early orthodontics on dentition and palate in severe palato-dental abnormalities.

2. To discern degree of growth of palatal and alveolar processes bordering the cleft after early orthodontic treatment.

3. Evaluate permanency of results in palatal and dental arch form after early expansion procedures in the presence of removable retainers and bone grafts.

4. Document character of bone graft, on a time basis and determine its contribution to palatal and dental habilitation in children with cleft lip and palate.

Methods Employed:

The data selected for this study were obtained on a group of patients ranging in age from 3 years to 7 years of age with cleft lip and palate. All received orthodontic therapy for 5 years - some in deciduous dentition and others in mixed dentition. Cephalometric, panorex, intra-oral occlusal X-rays were taken annually along with intra- and extra-oral photographs. Plaster models were made of patients' palato-dental abnormalities. All records reflected original problem and corresponding changes associated with orthodontic therapy.

Major Findings:

1. Early orthodontic treatment procedures proved effective in correcting palato-dental abnormalities - this was observed in mixed dentition.

2. Impacted palatal segments were unlocked after which time discernible growth of palatal and alveolar processes bordering the cleft was noted.

3. Palatal expansion to achieve acceptable palatal form was adequate and permitted bone grafting to be electively performed.

4. Bone graft restored integrity of dental arch and remained to allow unerupted teeth to emerge through it.

5. Maintenance of results were not complete after early orthodontics and retainers. Slight palatal collapse was recorded even in the presence of removable retainers and bone grafts.

6. Advantages of early orthodontics are clinically evident and outweigh reasons for delay. Early correction of malformed and malposed palates along with irregular dental arches result in more normal anatomical relationship of parts. It also contributes significantly to favorable growth for impacted palatal and alveolar bony processes adjacent to the cleft.

7. Bone grafting and retainers do not create permanency of results when utilized during early years of dynamic change inherent in palatal and dental structures. Their contribution is more critically challenged particularly in view of the fact that further orthodontics is needed to obtain the final desired palatal and dental form and position.

Significance to Dental Research:

Studies of this nature will produce some of the answers to questions of validity, and judiciousness in advancing early orthodontic and bone grafting procedures in cleft lip and palate habilitation. It could very well help decide the merits of surgical bone grafting procedures done during the early years of oral-facial growth and development.

Proposed Course of Project:

Patients will continue to be observed and treated until all permanent teeth are erupted and in occlusion - this is essential to prove the need of orthodontics, as a continuing process, for children with cleft lip and palate.

Part B not included.

Serial No. NIDR-104 (c) (68)
1. Oral Medicine and Surgery
2. Oral & Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Prenatal Development of the Larynx; Human and Comparative Investigation

Previous Serial Number: None

Principal Investigator: Dr. L. A. Krames

Other Investigators: None

Cooperating Units: Carnegie Institute of Embryology, Baltimore, Maryland; Department of Anatomy and Embryology, University of Toronto Dental School, Toronto, Canada

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

1. To review the literature and the Carnegie human slide material concerning the development of the larynx.

2. To apply autoradiographic, histochemical and fetal surgical techniques to many unanswered questions concerning the development of the larynx: e.g. Neural crest contribution to the laryngeal cartilages; Branchial arch contributions to laryngeal structures and epiglottis; Early laryngeal cartilage and muscle relationships; Epithelial fusion and separation of the Laryngotracheal groove.

Methods Employed:

1. Review of histologic material of normal laryngeal development in the human, rat, and chick.

2. Chick neural crest transplantations using tritiated thymidine for autoradiographic mapping.

3. Exteriorization of living rat fetuses into abdominal cavity of anaesthetized mothers for surgical access to branchial arch region.

Major Findings:

1. After comprehensive review of the literature it was evident that the material presented to date was incomplete, fragmented, and inconclusive. This was discussed with Dr. Ebert of the Carnegie Institute and it was his opinion that a comprehensive review article is indicated at this time.

2. In collaboration with Dr. Mac Johnston of the University of Toronto, using autoradiographic techniques, it was found that the laryngeal cartilages and connective tissue in the chick are neural crest in origin, demonstrating separate mesenchymal origins of muscle and cartilage in the larynx.

Significance to Dental Research:

It is clearly evident that laryngeal function is closely related to general oral and pharyngeal function and that basic knowledge of laryngeal development may shed light on the development of more cranial visceral arches.

Proposed Course of Project:

1. Further elaboration and documentation of neural crest contributions to the chick and possibly the rat larynx.

2. Autoradiographic mapping of 4th and 6th arch laryngeal structures in the rat with the development of fetal rat surgical techniques applicable to the larynx.

3. Anticipate publication of: a review article on the development of the Human Larynx; Neural crest contribution to the larynx in the chick; Application of fetal rat surgery to the development of the larynx.

Part B not included

Annual Report of the Dental Services Branch
National Institute of Dental Research
Summary Statement

As in previous years, the major objective or purpose of the Dental Services Branch is to render detailed service to both inpatients and outpatients of the National Institute of Dental Research. Dental Services Branch furnishes the clinical facilities for many NIDR investigators and the collaboration of our staff has served to enhance the clinical dental research program. In addition, our responsibility to provide optimum dental care for the research beneficiaries of the various categorical Institutes has been continued in an effective manner. Complete oral examination, evaluation, consultation and dental therapy is performed at the request of the patient's attending physician. The Dental Services Branch staff, composed of six professionals and twenty-three supporting personnel, is understandably limited in performing extensive dental care for all patients in the 500-bed complex; however, our staff is capable of performing all types of dental treatment which may be required.

The Branch has actively collaborated with all of the categorical Institutes in the discharge of our responsibilities to the total National Institutes of Health research effort. The following examples may be cited:

National Cancer Institute:

During the past year an increasing number of complicated, time-consuming to produce, maxillofacial prostheses have been constructed for patients with cancer. Prostheses of very high quality, which often defy detection even to the critical observer, are produced in almost routine fashion.

An efficient working relationship exists between the Dental and NCI surgery staffs. In one example of collaboration, a three-stage maxillofacial prosthesis is designed and fabricated for each patient undergoing surgical treatment for neoplasm of the paranasal sinus area. Surgical procedures for tumors of the maxilla or mandible usually leave the patient with a considerable defect resulting in the impairment of speech, mastication and deglutition. The objective in every case is to develop a prosthesis which will restore the anatomical defect, improve function and esthetics, thereby benefitting the general well-being of the patient.

In the post-surgical management of laryngectomized patients, the Dental Services Branch has developed a new tracheal prosthesis. These are one-piece, case-hardened pyrex glass appliances which are esthetic, hygienic, non-irritating and capable of maintaining humidity while protecting the trachea from debris. Approximately 75 patients are being maintained on these improved tracheal tubes with excellent response from the medical staff and the patients.

Participation in operations about the head and neck, including neck dissections, has been of mutual benefit to our staff and the maxillofacial surgeons. Suggestions and procedures by the dentist during surgery are of major importance to the ultimate success of the final prosthesis and the rehabilitation of the ora-facial region.

National Institute of Arthritis and Metabolic Diseases:

The development and fabrication of custom submaxillary saliva collectors for cystic fibrosis and normal control patients has provided a means to study the immunology and various chemical parameters of submaxillary saliva. The fundamental metabolic defect resulting in cystic fibrosis of the pancreas has not been determined. Study of the biochemistry and physiology of mucous and serous glands in all locations may provide the ultimate explanation for this disease.

National Heart Institute:

A greater number of dental treatments were performed on the patients of the National Heart Institute than for patients of the other Institutes. Cardiac surgery patients with congenital heart defects, or those requiring prosthetic valve replacement, pose problems of dental management in both the pre- and postoperative surgical periods. In the absence of proper dental care and preparation a simple procedure such as oral prophylaxis can precipitate a fatal, acute bacterial endocarditis. This program demonstrates clearly the importance of preventive care for patients who are to undergo or have undergone cardiac surgery. It is, therefore, imperative that candidates for cardiac operations obtain a thorough dental examination and completion of all necessary dental procedures before heart surgery in order to eliminate any possible sites of focal infection in the oral cavity.

Collaborative investigations were performed with the Cardiology Branch, National Heart Institute, in an effort to determine if there is an association between high arched palatal vaults and congenital heart disease. X-rays of the skull, teeth, and wrist and a cast of the upper arch were made on each study participant. The wrist films will be compared with accepted norms to determine bone age and to compare this with the chronologic age; the skull x-rays will be utilized for cranio-facial landmark measurements. Palatal vault measurements were taken of a group of patients with congenital heart disease and a normal control series of patients for comparison. All data obtained will be analyzed statistically for degree of correlation.

Extensive renovation of the Clinic was completed during this fiscal year. Three operatories were modernized to provide better physical facilities within the limited space available. The well-lighted easily maintained, functionally efficient suites reflect the thought and time given to their redesign, and to the seeking, developing and installing of the most advanced equipment available. These operatories now allow the dentist and dental assistant to be seated during four-handed dental procedures resulting in the performance of more dentistry for the chronically ill patient with less time and energy expended.

The renovation which was originally scheduled to be completed in three and one-half months experienced many delays and eleven months were required for final completion. This resulted in a loss of efficiency within the Clinic; however, the staff managed to perform an even greater number of treatments than in the previous year.

The Dental Services Branch staff is prepared to perform and has rendered all types of dental treatment found necessary for the patient examined.

The following table documents briefly the statistical facts of importance:

	Fiscal Year 1966	Fiscal Year 1967	Fiscal Year 1968 (Estimated)
Admissions to Clinical Center	4,195	4,076	4,023
Examinations	1,398	1,510	1,481
Visits	15,109	14,759	12,768
Treatments	36,546	31,259	32,460

Publications

1. Stanley, H.R., and Swerdlow, H.: Minimizing Pulpal Reactions With Prednisolone Therapy. The Effects of a Delayed Steroid Application To Cut Dentin. J. Oral Ther. & Pharm. 3: 1-8, July 1967.

2. Stanley, H.R., Swerdlow, H., and Buonocore, M.G.: Pulp Reactions to Anterior Restorative Materials. JADA 73: 132-141, July 1967.

3. Hamner, J.E., Lightbody, P.m., Ketcham, A.S., and Swerdlow, H.: Cemento-ossifying Fibroma of the Maxilla. J. Oral Surg. (in Press).

4. Gugler, E., Pallavicini, C.J., Swerdlow, H., and di Sant'Agnese, P.A.: The Role of Calcium in Submaxillary Saliva of Patients With Cystic Fibrosis. J. of Pediatrics. 71: 585-588, October 1967.

5. Stanley, H.R., Swerdlow, H.,: Prednisolone Therapy for Pulpitis. Dental World 22: Page 189, 3rd quarter 1967.

6. Gugler, E., Pallavicini, J.C., Swerdlow, H., Zipkin, I., di Sant'Agnese, P.A., Immunological Studies of Submaxillary Saliva From Patients With Cystic Fibrosis and Normals, Journal of Pediatrics (in press).

Published Abstracts

1. Pallavicini, J.C., Handwerger, S., Wiesmann, U., Swerdlow, H., di Sant'Agnese, P.A.: Albumin and Iga in Relation to Flow Rate in Normal Human Submaxillary Saliva, Federation Proceedings 27: p.253, 1968, 52nd Annual Meeting, Atlantic City, New Jersey.

2. Wiesmann, U., Pallavicini, J.C., Swerdlow, H., di Sant'Agnese, P.A.: Effect of Rate on Electrolytes and Carbohydrates in Normal Submaxillary Saliva, Federation Proceedings 27: p.676, 1968, Atlantic City, New Jersey.

3. Wiesman, U., Pallavicini, J.C., Handwerger, H., Swerdlow, H., di Sant'Agnese, P.A.: Effect of Flow Rate on Electrolytes, Carbohydrates and Proteins in Submaxillary Saliva of Normal Subjects and Patients with Cystic Fibrosis (CFP), Cystic Fibrosis Round Table Conference, Atlantic City, 1968.

4. Swerdlow, H.: Prosthetic Management of Maxillofacial Defects, Proceedings of the Third Joint Meeting of Clinical Society & Commissioned Officers Association of USPHS, San Francisco, California, March 25-29, 1968.

5. Stanley, H., Baer, P., Lightbody, P., Lundy, T., and Swerdlow, H.: Autogenous Reimplantation of Human Teeth, Proceedings of the Third Joint Meeting of the Clinical Society & Commissioned Officers Association of USPHS, San Francisco, California, March 25-29, 1968.

Presentation of Lectures, Papers, and Table Clinics Included:

1. Swerdlow, H.; Prosthetic Management of Maxillofacial Defects, Proceedings of the Third Joint Meeting of Clinical Society & Commissioned Officers Association of USPHS, San Francisco, California, March 25-29, 1968.

2. Stanley, H., Baer, P., Lightbody, P., Lundy, T., and Swerdlow, H.: Autogenous Reimplantation of Human Teeth, Proceedings of the Third Joint Meeting of the Clinical Society & Commissioned Officers Association of USPHS, San Francisco, California, March 25-29, 1968.

3. Swerdlow, H.: Newer Anterior Restorative Materials, 36th Annual Postgraduate Clinic of the District of Columbia Dental Society, Washington, D.C., March 10-13, 1968.

4. Swerdlow, H.: Maxillofacial Prosthesis, Postgraduate Seminar, Dental Assistants Association, USPHS Hospital, Baltimore, Maryland, June 9, 1968.

5. Hamill, M.R.: Fluid Resin Denture Fabrication, West Virginia State Dental Society, July 26, 1967.

6. Hamill, M.R.: Fluid Resin Denture Fabrication, Maryland State Dental Association, Baltimore, Maryland, September 20, 1967.

Serial No. NIDR-105 (c) (65)
1. Dental Services Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Reaction of the Human Dental Pulp to Cavity Preparations and Filling Materials

Previous Serial Number: NIDR-85 (c)

Principal Investigator: Dr. Herbert Swerdlow

Other Investigators: Dr. Harold R. Stanley

Cooperating Units: None

Man Years:

Total:	1/4
Professional:	1/4
Other:	0

Project Description:

Objectives:

1. To study the efficacy of cavity liners, base and restorative materials in maintaining and protecting the dental pulp.

2. To discover better ways to control the inflammatory reactions elicited by routine operative procedures.

3. To test new cement or restorative material capable of chemically and/or mechanically bonding to the tooth.

Methods Employed:

1. Patients selected for study must have non-carious, non-infected, vital teeth to be extracted for periodontal, prosthetic and/or orthodontic reasons.

2. Teeth are prepared with specific regard for the following: (1) rpms, (2) cutting tool, (3) coolant, (4) type and area of tooth to be used, (5) time intervals until extraction, (6) restorative material, and (7) cavity liner.

3. Teeth are extracted at varying intervals. After fixation, embedding, sectioning, and staining, a histologic evaluation is made and related to the clinical experience of the tooth.

4. The following experimental medicaments and restorative materials have been studied under the typical experimental design we have utilized in our previous work.

a. The toxic effects of newly developed adhesive materials (Addent 35, 3M Co.) and (Eastman Experimental Material, Dakor, L. D. Caulk Co.) which have the potential of supplanting the commonly used anterior restorative materials such as silicate and acrylic resin. These new direct dental filling materials are based on acrylic resins derived from epoxy resins. More than 50 percent of the binder is composed of chemically treated glass filler. An organic liquid catalyst polymerizes the binder in 3-5 minutes. These materials reputedly provide advantages such as low shrinkage during cure, high adhesive strengths, toughness, abrasion resistance and color stability.

b. Additional experimental work was completed to more clearly determine relative effects of cutting and grinding teeth "wet" or "dry". It has been suggested that the frictional heat developed in cavity preparation could be adequately controlled when using only air as coolant.

c. The use of a Copalite varnish under class V amalgam restorations is a continuation of the amalgam study published in 1962. In order to block the thermal, chemical and mechanical irritation of amalgam to the pulp tissue, two thin layers of varnish are applied to cavity walls.

d. A group of teeth have been used to evaluate the biologic compatability of a gallium-tin-palladium alloy developed at the National Bureau of Standards, Dental Research Section. The physical properties of gallium are reputedly superior to dental amalgam. The results are being published by Waterstrat at the Bureau of Standards.

e. A new temporary protective packing developed in Sweden was placed in twenty-five teeth using the experimental design described above. This product composed of calcium sulfate, calcium hydroxide and zinc oxide etc. has been reported to produce superior characteristics in sedating, sealing and insulating cavity preparations when compared to zinc-oxide and engeuol. This material (Pharmatec) was designed to function as a temporary filling material as well as a provisional adhesive agent for crowns and bridges. Presently, histopathologic analysis is underway.

Patient Material:

This year 46 teeth in 11 patients were utilized on these studies.

Major Findings:

1. If the health of the pulp is to benefit from the minimal pulp reactions associated with the new cutting methods, some treatment of the freshly cut dentinal tubules not lined by reparative dentin is imperative prior to restoration.

2. The application of the steroid formula inhibits the pulp inflammatory response to one of the more traumatic operative procedures. There is no evidence at this time to suggest that the application of this formula in this dosage is detrimental to the human dental pulp.

3. Reparative dentin was found at approximately the same time interval and occurred at a similar rate as seen in control teeth. This indicates that healing was not interfered with in the experimental group.

4. The pulpal response of 293 intact human teeth were evaluated to compare several new composite anterior restorative materials (with and without liners) to zinc oxide-eugenol and silicate cements. It was found that the initial pulpal reactions created by the Eastman product were similar to silicate restorations. Addent 35 without a liner was initially less irritating than silicate. The pulpal response intensity subsided with the Eastman product after extended postoperative intervals, whereas the lesions produced by the 3M product became more severe. The vinyl-copolymer liner recommended by the 3M Company was not adequate to protect the pulp tissues from the irritating properties of addent 35 filling material.

5. Those experimental teeth prepared with only air cooling developed significantly more severe pulp pathology than a comparable group of teeth prepared with a water-spray coolant. The air-cooled teeth demonstrated a high percentage of pulp lesions exhibiting burn characteristics, such as lesions extending beyond cut tubules, massive reactions leading to intra-pulpal abscesses and an increased rate and amount of reparative dentin. Therefore, if pulp damage is to be reduced to a minimum when a high-speed handpiece is used, adequate water spray should be used during cavity preparation.

Significance to Dental Research:

1. The development and acceptance of a restorative material, cavity liner, or base material must not only be governed by adequate measures of evaluation for physical properties, but must also conform to rigid biological requirements. The maintenance and protection of the dental pulp and supporting structures is a fundamental principle in restorative dentistry.

2. The baseline data accumulated provide the ability to evaluate properly any restorative material introduced for dental procedures.

3. The evidence now available from numerous histopathological studies supports the following recommendations as guidelines for minimizing pulpal trauma in restorative dental procedures.

a. Treat all teeth as potentially "sick".
b. Keep the cavity shallow in dentin.
c. Speeds above 50,000 rpm are more biologically compatible.
d. Use a water coolant when cutting tooth structure.
e. Exert light force to exposed dentin.
f. Use smaller cutting tools at higher speeds.
g. Keep irritating drugs away from exposed dentin.
h. Use zinc oxide and eugenol on dentin when possible.
i. Delay final setting of restoration, when possible, for secondary dentin deposition.
j. Sedate, seal and insulate all cavity preparations.

Proposed Course of Project:

Investigations of the following:

1. Rationale for the use of corticosteroids to arrest an established inflammatory reaction.

2. Comparative toxic effect of experimental adhesive restorative materials (Addent, Dakor, Experimental Epoxy).

3. Post-extraction cavity preparation for observation of displacement of odontoblasts.

4. Cavity preparation response in teeth with incipient carious lesions.

5. Silicate - an anterior restorative material.

6. Full crown preparations vs. the vertical parallel pin preparation.

7. Cementing procedures.

8. Horizontal pin-lay.

9. Gold foil vs. amalgam condensation.

10. Evaluation of reduced temperature of the water coolants used in cavity preparation.

11. The value of a varnish cavity liner under amalgam restorations to reduce the established trauma from amalgam insertion.

12. The value of new temporary filling materials improving, sedation, sealing and insulation.

Part B

Publications

1. Stanley, H. R., Swerdlow, H., and Buonocore, M. G.: Pulp Reactions to Anterior Restorative Materials. J.A.D.A. 73: 132-141 July 1967.

2. Stanley, H. R., Swerdlow, H.,: Prednisolone Therapy for Pulpitis. Dental World 22: Page 189, 3rd quarter 1967.

Serial No. NIDR-106 (c) (68)
1. Dental Services Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Palatal Vault Measurements in Patients with Congenital Heart Disease

Previous Serial Number: None

Principal Investigator: Dr. Barry Goldman

Other Investigators: None

Cooperating Units: Dr. Lawrence S. Cohen, Cardiology Branch, NHI, NIH

Man Years:

Total:	1/4
Professional:	1/4
Other:	0

Project Description:

Objectives:

Preliminary observations suggest that patients with congenital heart disease have a high incidence of high arched palates. This is a pilot study to determine if an unusually high arched palate is characteristic of patients with congenital heart disease.

Methods Employed:

Patients with documented congenital heart disease from the Cardiology Branch of NHI are utilized. Dental radiographs, a growth and development chart, wrist radiographs, and an impression of the maxillary arch are obtained for each patient.

Accurate palatal measurements are made on the stone cast to determine the Palate Height Index. This index will be compared to that of normal control patients and analyzed for statistical correlation.

Major Findings:

This study is presently in progress and findings have not been completed.

Significance to Dental Research

Previously the association between high arched palate and congenital heart defects has been based on subjective observations of the palate. This study will obtain valid palatal dimensions in congenital heart patients. A significant correlation may be a useful tool in the diagnosis of congenital heart disease.

Further study may also demonstrate a relationship between congenital heart defects and other factors such as cranial growth and development or orthodontic abnormalities.

Proposed Course of Project:

Twenty-five to fifty patients will be included in this pilot study. To date, nineteen patients have been examined.