ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL INSTITUTES OF HEALTH

1968-1969

NATIONAL INSTITUTE OF DENTAL RESEARCH

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ANNUAL REPORT

July 1, 1968 - June 30, 1969

US NATIONAL INSTITUTE OF DENTAL RESEARCH

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REPORT OF THE DIRECTOR

THE NATIONAL INSTITUTE OF DENTAL RESEARCH

July 1, 1968 - June 30, 1969

by

Seymour J. Kreshover

After a period of growth which saw the appropriations provided to the National Institute of Dental Research rise from $10 million in FY 1960 to $30 million in FY 1968, the year ending June 30, 1969, was one of holding-the-line. While almost $30 million was appropriated for FY 1969, the Revenue and Expenditure Control Act of 1968 resulted in a cutback of $548,000 of which $270,000 was subsequently released to pay salary increases as required by Section 212 of the Federal Salary Act of 1967. After all cutbacks and adjustments, the net funding available for NIDR operations totaled $29,415,000 in 1969 as contrasted with a comparable total of $29,027,000 in FY 1968.

Another significant turn of events in FY 1969 occurred with respect to full-time permanent staff of the Institute. Simultaneously with the increase in appropriations, the full-time permanent staff had grown from 151 in 1960 to 322 in 1968. However, the latter number was reduced to 301 in FY 1969 under the requirements of the Revenue and Expenditure Control Act of 1968 which placed certain ceilings on Federal employment.

The two aforementioned restrictions imposed on the Institute as its share of an overall program to reduce Federal expenditures and employment, generated an intensive self-evaluation as to program and staffing priorities within the Institute. Among the determinations made were to continue the Caries Task Force effort as a top program priority and to maintain the categorical extramural programs at their authorized staffing level through, as it became necessary, the reassignment of intramural staff.

Reorganization of the National Institutes of Health gave impetus to an extensive study and analysis of the dental functions incorporated in the new NIH structure and as reflected by NIDR and the Division of Dental Health. This effort included the utilization of a group of prominent dental and medical educators-researchers in the provision of expert and authoritative advice and assistance from the outside community of dental and biomedical education and research. At the conclusion of the study, a formal report with recommendations as to the organization of dental functions within the expanded NIH was submitted to the Director of NIH for his consideration.

As a means of improving communication between NIDR and the dental schools of the country, a series of meetings with dental school deans and their research coordinators were undertaken. A typical meeting involved the

representatives of from six to eight dental schools and the top staff of NIDR in a relatively unstructured discussion of the relationships between the schools and NIDR, the identification of problem areas, and the action which might be taken to resolve problems to the mutual satisfaction of all interested parties.

A substantial amount of staff time and energy was devoted to the consideration of ways and means whereby NIDR could more effectively mount applied research programs, and thereby increase its contribution to the national goal of improving the health of all Americans. Special emphasis was given to the preparation of state-of-the-art reports which would provide authoritative guide-lines not only as to program relevance of research grant applications but also as to organized programming of research, through such approaches as research contracts, in order to quickly follow-up and exploit promising leads.

The controlled fluoridation of public water supplies continues to be fraught with controversy. A leading national periodical carried stories which focused on the problems of dialysis when fluoridated water is used and implied that the full facts on fluoridation had not been disclosed by the Public Health Service. With the collaboration of the National Institute of Arthritis and Metabolic Diseases and of the Division of Dental Health, a statement was prepared for and signed by the Surgeon General which reiterated the complete safety of controlled fluoridation of public water supplies.

In the interest of brevity, other matters such as organizational changes and planning for the future will be covered in the reports of the Director of Intramural Research and the Associate Directors for Extramural Research, Special Programs, and Program Planning and Evaluation.

Office of Program Planning and Evaluation

During FY 1969, the Office of Program Planning assumed the additional responsibility for assessing the Institute's program activities and was renamed the Office of Program Planning and Evaluation. Fulfillment of the duties concomitant with this new function were facilitated by the appointment of an Assistant to the Associate Director and by the organizational assimilation of the Reports and Analysis Section. This arrangement provided for a more comprehensive coverage of NIDR's activities and progress, and made a direct contribution to the over-all planning role of the Office.

Of the special projects that were undertaken during the year, the following list illustrates the variety and scope of recurring and ad hoc reports and publications produced by the OPP&E:

(1) Through its efforts to establish a sound base of data, one which could be comparably analyzed from year to year; this Office produced the first annual National Institute of Dental Research Grants and Awards publication. The report covers all research grants, training grants and other awards made from FY 1967 funds.

(2) An in-depth analysis of one of the extramural programs, Periodontal Diseases and Soft Tissues, was carried out to determine the data and data arrays appropriate for an automated information system. In covering all aspects of the periodontal program -- research grants, training grants and awards, this report also served as a prototype for planning similar analyses of the other categorical extramural programs. Various graphic devices and a series of indexes to highlight and locate salient points will continue to be used to characterize program scope and direction, and to place each program in perspective to the entire extramural effort.

(3) Following a proposal made by this Office in 1965 -- that a worldwide survey of institutions engaged in cleft lip/palate research, treatment, and rehabilitation as an aid in evaluating and strengthening the NIDR program of support in this field -- its collaborative efforts produced the NIDR Directory of U.S. Facilities Providing Cleft Lip and Cleft Palate Services was produced in collaboration with the oral-facial growth and development program. The 300-page report provides details on 244 institutions such as address and telephone number, kinds of services provided, number of patients served, professional staff, and an indication of whether there are research and professional training activities.

(4) With the objective of aiding administrators and investigators in identifying and locating young scientific talent for careers in dental research, the Office produced and distributed a booklet showing the trainees and fellows trained under NIDR support during 1968. Besides specific information on the trainees and fellows, the listing includes the names and addresses of the program directors and sponsors who may be contacted for further information.

(5) Following the annual site visits and prior to the meeting of the Dental Research Institute and Special Programs Advisory Committee, an analysis was made of the Dental Research Institutes. Factors evaluated included the degree of conformity with the basic guidelines, site visitor comments, fiscal history and peripheral support, staff size and qualifications, existence and composition of advisory bodies, and space available with percentage of occupancy.

(6) As a reference tool containing data on various aspects of the dental health programs in the Federal government, a Manual of Federal Dental Programs was compiled. Organizational and descriptive material was solicited from each segment of the Federal government that could be identified as a contributor to or participant in the Nation's dental health effort along with figures on professional manpower and levels of support for research, education and services.

(7) Facts and figures pertaining to the many aspects of health economics as related to dental research are being collected in the form of a handbook. Intended for internal use in determining the cost benefits of dental research, the handbook contains published reports on the occurrence of dental disease and its costs in terms of money, time and manpower.

Further data and information backup for the Office is expected to come from the Institute-supported Dental Research Information Center (DRIC), operated by the American Dental Association. The Center launched a resurvey of the dental investigator community and is developing basic files of information on dental research in the areas of personnel, active projects, and institutional resources. Analysis of this data can be performed to identify trends, developments, and accomplishments in dental manpower and research. The first product of this activity will be a directory of dental investigators -- complete with name, institutional address, degrees, and area of research interest.

Information Office

Concern with health in the United States today transcends the major killing and crippling diseases. In a recent survey, more than three of every four people indicated that needed dental care should be provided free of charge to those who cannot afford it. This growing awareness of the importance of oral health, coupled with the awesome dimensions of dental problems, place new urgency on the communication to the public of information on advances in the prevention of oral disorders. Similarly, dental practitioners need to be well-informed of the newest research findings, in order to cope with the dental needs of an increasing population.

Exploring ways of reaching large groups of people through radio and television, an increasingly important communications channel, the NIDR Information Office was instrumental in introducing a new NIH-wide radio series over WGMS. Entitled "Discussion: NIH," the program features interviews during the intermission of the Library of Congress concerts on Friday evenings. During the year, ten NIDR representatives have appeared on this series. NIDR has also participated on five of the programs in the NIH television series, "NIH Reports" over WRC-TV. In addition, a new effort has been launched by NIDR to feature research reports of grant-supported scientists on radio and television in various parts of the country. Two such interviews, presented during the year over a radio station in Miami, and a TV station in Boston, drew much favorable comment. Also, an NIDR investigator appeared on a TV interview program in San Francisco. In addition, the NIDR Information Office and two members of the extramural staff participated in a closed circuit television program at the annual meeting of the American Dental Association.

Film clips of NIDR-supported research were transmitted by ABC in Washington, D. C.,to its ninety-five television affiliates throughout the country. Interest triggered by other distribution of these clips resulted in coverage of an NIDR research project on dextranase on the "Today" NBC network show. In addition, the tape was fed audiovisual on the NBC News Program Service, which encompasses 108 television stations, as well as on the Afternoon Special Report, with a coverage of 192 radio stations.

As a result of media interest generated by the June 1968 press briefing held in conjunction with the NIDR's 20th anniversary, a substantive article on dental research appeared in the July 8 issue of U.S. News and World Report.

Stimulated in part by periodic mailings of dental research information to selected science writers, other articles appeared throughout the year, in

addition to dental publications, in such periodicals as This Week (a national Sunday supplement); Good Housekeeping; American Professional Pharmacist; Our Navy; American Engineering; Electronics Age; Business Week; Journal of American Veterinary Medicine; Journal of the American Medical Association; U.S. Medicine; Medicine and Hygiene; and Better Homes and Gardens. Known articles in the foreign press appeared in British, French, Italian, and Australian Journals, and several Latin American publications.

Publications for student groups of various ages have also been responsive to this flow of information. These groups represent, by and large, present and future consumers of dental research information and potential dental scientists who will further enlarge the body of knowledge and contribute to the prevention of oral disorders. Among such publications, the 1969 issue of the World Book Encyclopedia will carry an article on the relatively new fluoride gel method of topical application. Information on recent advances in dental research was also provided to the Americana Encyclopedia, to the Britannica Yearbook of Science and the Future, to a high school textbook, Foundations of Biology by Trump, Volker, and Oakes, and to a book being prepared on science in dentisty as part of McGraw-Hill's "Young Scientist Series." Younger children were reached through such publications as Science News, Nature and Science, My Weekly Reader, and You and Your World. NIDR research reports also covered 4 pages of the November issue of Current Science, the American Education Publication for school children.

In a more formalized and targeted effort to attract young science-oriented students to dental research, the NIDR produced, under grant, a half-hour, color motion picture entitled "Laboratory of the Body." Premiered at the annual meeting of the American Dental Association last fall, the film, whose scripting and production was closely supervised by the NIDR Information Officer, is directed toward science-oriented high school seniors and college freshmen, career guidance counselors, and science teachers. A flyer, "This is Dental Research," was also prepared for distribution to students viewing the film and for use in responding to inquiries. A more definitive dental research career publication for guidance counselors and science teachers is now in preparation.

As the initial promotional effort, an order card describing the film, together with the flyer, was sent to high school and college science teachers, and career guidance counselors in the United States, using the mailing lists of the National Association of Science Teachers and the American Personnel and Guidance Association. The response for prints was so overwhelming that an additional 50 prints of the film were made, making a total of 125 prints. Some 1,300 bookings of the film were made during the 1968-1969 school year, and requests for approximately 2,000 unfilled bookings will be carried over to September 1969.

In addition to its potential value in attracting young science students to dental research, the film has served as a valuable public information function. It has made thousands of students as well as their science teachers and guidance counselors aware of the broad scientific base of dentistry, and has hopefully made them more receptive to the application of research advances.

An unexpected degree of response to the flyer alone has resulted in the receipt of approximately 430 inquiries requesting additional information on dental research, which has added to the already burgeoning number of public inquiries being received. For example, approximately 2,270 public inquiries were handled this year by the Information Office staff, as compared to 646 in 1965.

The Information Office continues to prepare and disbribute Abstracts from NIDR Scientists to dental scientists throughout the U. S. to keep them informed of the increasingly extended range of Institute activities. A large mailing list is maintained for making available Institute scientific reports to dental researchers outside the United States. Through this mechanism, 7,200 pieces of literature were mailed in FY 1969. In addition, and in cooperation with the dental advisor to the Pan American Health Organization, 800 reprints of clinical interest were mailed to practicing dentists throughout Latin America.

This year the Information Office revised three leaflets relating to the extramural programs. One pertained to the Institute-supported summer research opportunities for dental students in 1969. To the college addresses of over 14,000 dental school students, 21,818 pieces of training and fellowship literature were mailed.

Internal reports prepared in whole or in part by the Information Office during FY 1969 numbered approximately 141. 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 include the Director's Opening Statement, narratives for annual National Science Foundation reports, highlights of research progress, and other Congressional reports. Also, six speeches were prepared by the Information Office staff, and 52 articles, press releases, summaries and announcements were produced.

The Information Office, responsible for the publicity for the 1968-69 Combined Federal Campaign at NIH, blended science with art in this year's approach. Following a survey to identify troublesome points in previous campaigns, the Information Office staff prepared a series of materials designed to puncture the "myths." An appeal from Sam the Hamster, depicted in an engaging cartoon and photograph on leaflets, billboards, tent cards, and posters, plus an appearance on WMAL-TV and WGMS radio, through his spokesman, the Director of NIDR, who served as Chairman of this year's campaign, brought an enthusiastic response from NIH employees to the needs of the many charities funded by the DFC. The campaign exceeded 92 percent of the assigned quota with a total gift from NIH of $183,303.

An important part of the Information Office's responsibility is to keep the dental practitioners well-informed of the newest research findings. This year a continuing service of the office provided 3,468 monthly news features to dental editors. These articles, carried in the dental journals, bring to the practitioner findings which are important to the oral health needs of the nation.

NIDR exhibits were displayed at 8 scientific meetings during FY 1969. One of the exhibits "Case Studies of Oral and Pharyngeal Form and Function" won an award for "excellence of scientific presentation" at the meeting of the American Speech and Hearing Association. Information Office staff manned the Institute's exhibit "Spectrum of Career Opportunities" at the American Dental Association meeting and the American Personnel and Guidance Association meeting. Approximately 9,000 pieces of literature were distributed from these exhibits.

The Information Office staff again assisted the American Dental Association in publicizing the 21st observance of National Children's Dental Health Week. An article was prepared for distribution to 445 house organs interested in science, with a potential reading audience of 13 million; arrangements were made for the NIDR Director to be interviewed in New York on the World Today program (Mutual Radio Network) and on NBC's Monitor; an NIDR investigator appeared on a local (Washington, D.C.) children's television program; and a cartoon promoting a "Smile-In" and a brief write-up were supplied to the NIH Record. Also, local radio programs carried brief spot announcements on Children's Dental Health Week. These announcements were prepared by the Information Office staff in cooperation with the NIH radio coordinator.

The Information Office was extensively involved this year in the editing, publishing, and promotion of a 49-page booklet entitled "Barnacle Cement as a Dental Restorative Adhesive." This publication presents a survey of the progress to date in this targeted research program. Similarly, comprehensive editorial review and publication arrangements with the Government Printing Office were handled by the Information Office for the 600-page "Dental Science Handbook." This handbook will be widely publicized and distributed to non-dental scientific and industrial audiences to promote better understanding of research in the dental sciences.

The Information Office acts as a clearance center for manuscripts produced by Institute scientists. During the past fiscal year, 114 manuscripts and 54 abstracts were presented for clearance. The Office also prepared the NIDR component of the NIH annual bibliography, and "The Professional Staff Listing of NIDR."

The always important "Visitor Program" of the Information Office continued to be active this year. Considerable staff time was required to arrange suitable programs for individuals, as well as for groups such as Naval and Army dental officers, pedodontic study clubs, dental hygienists and assistants, and students. Approximately 317 persons visited the Institute this fiscal year, in addition to visits arranged and handled by individual professional staff members.

Attempts to enlarge the pool of people knowledgeable in dental research will continue to be a major focus of information program activities in FY 1970. This project has a two-fold purpose: (1) to increase the seriously deficient corps of dental scientists, and (2) to increase awareness of oral health problems by the general public and to encourage the application of research advances in coping with them.

Dental Research Institutes

The Dental Research Institutes Program has made a significant and rather impressive beginning during the 22 months that have elapsed since the first awards were made. A major portion of this time has been devoted to administrative organization, development of operational guidelines, recruitment of scientific personnel, program planning, acquisition of equipment, and program evaluation. The success thus far achieved by the universities in accomplishing these fundamental activities is most gratifying. This is especially true in view of the problems usually associated with the implementation of broad-gauged collaborative efforts. Although the period of actual operation of the Institutes has been very short, the great potential of this innovative program has become readily apparent.

The task remaining is to bring to fruition a vigorous, productive program which will broaden the dental research and graduate training base and enrich the environment in the nation's dental schools. This will require a continuing assessment of the programs of the individual institutes so that modification can be initiated as required.

The first comprehensive annual evaluation was recently completed at each of the Institutes and included an internal (university) review and an external (NIH) review. The results of these reviews were discussed with appropriate officials at each university and several beneficial modifications have already been made.

Inadequate funding continues to curtail the orderly development of the Institutes Program. Although commitments were strongly implied to some nine or ten universities, only five have actually received awards for the establishment of Dental Research Institutes. Furthermore, NIDR will be unable to support these five at the approved levels during FY 1970.

The joint commitment of the Federal Government and the universities to this national program is clear, and it is therefore expected that the FY 1971 budget will include adequate increases for its support.

REPORT OF THE EXTRAMURAL PROGRAMS

NATIONAL INSTITUTE OF DENTAL RESEARCH

July 1, 1968 - June 30, 1969

by

Dr. R. M. Grainger
Associate Director for Extramural Programs

The NIDR Extramural Programs faced appreciably greater financial difficulties in initiating and maintaining research projects this year than in FY 1968. It was mentioned in last year's Annual Report that the Extramural Program's demonstrated potential for growth was limited by the funds available, that it was impossible to fund all the highly meritorious new research grant applications approved by the NADRC, and that only one supplemental application was funded. A more severe situation existed this year. The more stringent budget allotted to NIDR-EP in FY 1969 made it impossible as a general rule to fund type 2 research grant applications having priority scores lower than 250. New applications having priority scores of less than 215 could not be funded in the absence of unusually high program relevance. Only one supplemental award could be made.

At the end of FY 1969 it is estimated that NIDR-EP will be supporting 271 research projects as compared to the 303 projects active in FY 1968. Furthermore, due to lack of funds, the Institute has had to administratively inactivate 38 new, competing and supplemental applications in the amount of $995,000 (direct costs only). In addition, 44 applications will be maintained on the potential payment list into the next fiscal year. These latter applications have a direct cost value in excess of a million dollars.

Only by assiduously negotiating research grant budgets downward was the Institute able to finance 271 projects. Approximately $800,000 was accumulated by negotiating the budget of the new, competing and non-competing applications with each domestic principal investigator by telephone. The money so saved was used to fund new and competing applications which had excellent priority scores and high program relevance. It is recognized that accumulating negotiated savings of this magnitude can only temporarily slow the shrinking of the research grant base.

As a result of additional criteria regulating institutional eligibility for General Research Support Grants, approximately 18 dental schools will fail to qualify. An important function of these grants has been the support of pilot studies conducted by young investigators. In part as remedy for the cessation of such support in ineligible dental schools, and in part as a means of assuring qualified junior investigators additional opportunities to undertake quality

research in the basic and clinical sciences relevant to dentistry, this Institute proposes to establish a Dental Small Grants Program (DSGP). One or two year renewable research grants in an amount not to exceed $10,000 plus indirect costs would provide funds for small items of equipment, supplies and technicians' salaries. In the main, these grants would be used to develop and test a new technique or method, to analyze data previously collected, to perform pilot studies, or, in general, to carry out well circumscribed investigations of limited duration. Funds could not be used for defraying travel costs, or publication costs except in unusual circumstances. They also could not be used for dissertation expenses, supplements, or interim support for projects which are already funded or under review by granting agencies. Applications would receive primary review three times a year, usually in January, May and October. Initially the Dental Program Project Committee would undertake the scientific review. Consideration will be given to the possible advantages of making the deadlines for receipt of DSGP applications more flexible and setting up a separate committee for primary review. The program would be supported by regular research grant funds, with a maximum amount of $200,000 earmarked for the program in its initial year. This program will be maintained for a minimum of five years to provide sufficient stability and to permit a sound evaluation of its effectiveness.

The question of referral guidelines remains an important issue. This Institute continues in its efforts to obtain clarification of the guidelines and to resolve areas of overlapping interest with other granting organizations.

The Institute has continued to assess its research manpower training activities to better assure maximum productivity. For example, a two-day workshop on the role of the biophysical scientist in the dental school was held in Atlantic City in February. The 90 participants included the dental school deans or their delegated representatives, the NIDR-supported biophysical science training program directors, and their advanced students. A report of the workshop is planned for publication in the summer of 1969.

In recognition of the need to expand the number of high quality clinical research training programs, NIDR appointed an Ad Hoc Committee on Clinical Research Training which held its first meeting in February in Bethesda.

State-of-the-Art studies are essential to a meaningful programmatic effort. Their significance again is magnified by the present shortage of funds, since it is increasingly important to invest available resources in a knowledgeable manner. Three meetings related to this question have been sponsored by NIDR this year.

In summary, the estimated level of program area activities as of June 30, 1969, with respect to the number and total dollar value of active grants administered is as follows:

Program Area	Research Grants		Training Grants		RCDA Grants		Fellowship Grants	
	No.	$(000s)	No.	$(000s)	No.	$(000s)	No.	$(000s)
(1) HT	77	$3,756	27	$1,276	11	$196	16	$109
(2) ST	76	2,450	25	1,327	24	470	42	473
(3) GD	77	2,781	31	1,609	12	233	20	194
(4) MS	41	1,500	18	1,111	2	37	2	23
	271	$10,487	101	$5,323	49	$936	80	$799

(1) Dental Caries and Hard Tissues Program

(2) Periodontal Diseases and Soft Tissues Program

(3) Oral-Facial Growth and Development Program

(4) Materials Science and Special Clinical Studies Program

The scope of Extramural activities is illustrated by selected examples for each of the categorical program areas, which are summarized in the following pages.

Dental Caries and Hard Tissues Program

This categorical area is concerned broadly with research on dental caries and hard tissues, and focuses essentially on bone, tooth substance, plaque and calculus. Projects include investigations of the physical and chemical properties of normal and pathological bone, dentin, enamel and cementum as well as their metabolism. Although coronal caries is the major disease condition in our area of responsibility, we are interested also in other defects such as fluorosis, hypoplasias, erosion, attrition, abrasion and root caries.

Dental Caries

In spite of the restriction on monies for the support of research, more emphasis will be placed on caries investigations in order to capitalize on the findings from recent studies which are rapidly bringing us closer to the reality of caries prevention. There is growing evidence that caries on smooth surfaces, root surfaces, and in pits and fissures are not the same entity, require different sets of etiological circumstances, and must be considered separately by the investigator.

To focus interest on caries research, a variety of priority approaches have been identified, all of which seek ultimate caries prevention through interference with some part of the interlinked chain of host, dietary, and microbial causative factors. These include efforts to 1) increase the resistance of enamel to tooth decay, 2) develop the host's immunity to caries, 3) eliminate the cariogenic potential of foods which act as substrates for caries inducing micro-organisms, 4) enhance the pre- and post-eruptive cariostatic influences of foods on teeth, 5) eliminate or otherwise control the formation of tooth plaque, and 6) develop antibiotic approaches for elimination of cariogenic microorganisms.

An example of research directed at increasing the resistance of enamel to the initial caries attack is the work being carried out at the Forsyth Dental Center. Scientists there have found that more fluoride is incorporated into enamel by topical application if the teeth are first treated for one minute with dilute phosphoric acid or a solution of an aluminum or titanium salt. The same team of investigators also developed a method for biopsying enamel to determine the uptake of fluoride following treatment. The procedure is safe, quick, and reliable and removes a layer of enamel only 1-2 microns thick which can then be analyzed for fluoride or calcium by means of a fluoride electrode and atomic absorption spectrophotometry.

Another dental scientist at Tufts University, School of Dental Medicine is attempting to develop a caries susceptibility test. Samples of saliva and plaque are being collected from caries-free and caries-active individuals and subjected to a battery of 18 tests to try to identify chemical differences between the samples collected from the two groups.

At the Massachusetts Institute of Technology a group of investigators is using a sophisticated method for controlling the diets of pregnant and lactating mother rats to study the effects on teeth of minerals such as manganese, fluoride and phosphates. Also under study in experimental animals are other factors such as the relationships of diet composition, diet restriction, and frequency of eating to kinds and amounts of lipids and fatty acids in the animals' teeth and the development of dental caries.

Microorganisms which cause decay on smooth surfaces of the teeth form extracellular polysaccharides which are thought to give plaque their ability to adhere to smooth enamel surfaces. Now it has been found by scientists at the Forsyth Dental Center that these polysaccharides are made up of large molecules of dextran or levans with a molecular weight too high to be soluble. By investigating the enzyme responsible for making these high molecular weight materials, it was found that the enzyme dextran sucrose could be diverted from synthesizing high molecular weight insoluble dextrans by incorporating alternate glycosyl acceptions such as low molecular weight dextran fragments in enzyme-sucrose reaction mixtures. The addition of low molecular weight dextran to sucrose broth cultures of cariogenic organisms in an in vitro model system was found effective in preventing the formation of plaque-like deposits. When, in later experiments, these compounds were incorporated into the diet of hamsters infected with Streptococci Mutans, only minor plaque deposits developed, and little or no caries occurred.

Root caries, which often result in early pulp exposure and which are difficult to restore, appear to be of increasing concern to the dental practitioner. Yet little is known about their prevalence, distribution or etiology. With support from NIDR, a group at Temple School of Dentistry has undertaken the task of establishing a differential diagnosis and determining the public health significance of the lesion. Later they hope to extend their work into a study of possible etiologic factors.

Using fluorescent antibody technics, a group at the University of Miami has identified from humans several strains of organisms which are capable of producing caries in experimental animals. In addition to developing technics to relate caries activity to the percentage and distribution of these organisms in selected groups of children, the studies include an assessment of the amount and frequency of consumption of cariogenic foods and the oral hygiene status of the individual.

Scientists at the Eastman Dental Center have developed technics which yield reproducible plaques in repeated samplings. They also have standardized their technics for measuring plaque and now are ready to launch an in vivo study of the inherent variables of plaque to find the greatest differentials between caries active and caries inactive subjects. Studies under these standardized, well-controlled conditions will permit a comparison of the acid producing capability of plaques from subjects with varying rates of caries activity.

Hard Tissues

In a study of hydroxyapatite combining the use of neutron diffraction, x-ray diffraction and NMR, a grantee at Georgia Institute of Technology has described the detailed mechanism by which small amounts of fluorine can inhibit diffusion in hydroxyapatite. Fluorine is hydrogen-bonded to the hydroxyl oxygen, producing OH-F and OH-F-HO groups which in turn are bound to the rest of the structure with the strength of the fluorine-calcium bonding. These groups then may be expected to inhibit diffusion along the hydroxyl ion column. A parallel study using neutron diffraction substantiated and quantified the finding that significant amounts of hydrogen are missing from the hydroxyapatite-like structure of human dental enamel, thus permitting the possibility of substitution of significant quantities of ions such as F- or CO_3.

At the University of California at Los Angeles, NIDR-supported researchers have developed a technic which has been 90% successful in inducing bone formation. The process involves decalcification in dilute hydrochloric acid (at 2°C) of a sample of bone, dentin or cartilage, then freeze drying (lypholization), followed by irradiation. Implants of the lypholized bone result in slight inflammatory action followed by bone formation within two weeks. Research is presently underway by an interdisciplinary team to characterize, localize and isolate this bone-induction principle and to determine the mode and site of action.

In a study of the composition and formation of calculus, an NIDR grantee at Columbia University School of Dentistry reported that the deposits found on the lingual surfaces of the lower anterior teeth differ in composition from those found in the rest of the mouth. Differences also were demonstrated in the composition of plaque from subjects classified as heavy and light calculus formers. Plaque from the heavy calculus formers had higher calcium, phosphorus and protein content but lower amounts of carbohydrate. The data further suggested that differences in calcificability are apparent as early as 48 hours and that calcification seems to be associated with relative increase in protein and decrease in carbohydrate. It was also observed that the rate of formation of subgingival deposit was much slower and less in amount than that of supragingival deposit. Histologically, however, the supra and subgingival deposits were indistinguishable. Because different fluid environments are involved (saliva versus crevicular fluid plus inflammatory exudate), the study will be extended to characterize both gingival fluid and salivary secretions, and to determine if chemical differences are present.

At the University of Illinois, NIDR-supported scientists observed the effects of large parenteral doses of NaF or $SrCl_2$ on rat incisor dentin. Using the electron microscope and examining at various intervals after injection, they found no odontoblastic disturbance but several definite differences in the abnormal matrix induced by these ions. Small dense granular deposits were observed in the predentin following the injection of strontium but similar deposits were not produced by fluorine injection. Mineralization of the fluoride-induced hypomineralized zone, although slowed, proceeded in the usual collagen-oriented pattern. But mineralization of the strontium

response followed an abnormal pattern in that patches of crystalline material accumulated with little relation to the collagen fibrils. The observation that unmineralized areas remained within the hypomineralized region, along with the former observations that mineralized areas within this region contain large crystals, supplies evidence for an inhibition of nucleation.

Periodontal Diseases and Soft Tissues Program

This program area is concerned primarily with four categories of illness: periodontal disease, salivary gland disorders, oral ulcerations, and oral neoplasms.

Periodontal Disease

The rapid progress in medical science and technology during the past half century has been effective in eliminating many of the death-dealing diseases of man. This progress has resulted in greatly improving the potential for human health, comfort and longevity. However, much of the Nation's population that is living longer is plagued by chronic and debilitating ailments, and foremost among these in frequency is periodontal disease.

Today periodontal disease is regarded as multifactorial in origin and probably represents more than one discrete or characteristic disorder. While much has been learned about pathogenesis, the many remaining gaps in knowledge make it difficult to project any early approach to completely satisfactory assurance of control and prevention.

It is evident that a basic understanding of periodontal disease requires first an understanding of the normal periodontium as well as the relationship of the component tissues to the various microorganisms inhabiting the oral cavity and the complex aqueous environment. Thus, some 20 currently supported research projects deal with the normal anatomy and physiology of the periodontium. In general, these studies are concerned with the microscopic and ultrastructure of the periodontal tissue, blood and lymph flow, nerve function, biochemistry of the gingiva, and collagen.

One of the key tissue substances in the periodontium is the complex protein, collagen, whose fibers act as anchor cables and shock absorbers of the teeth in their bony sockets. As reported in the Intramural Research section of this Annual Report, a significant achievement by NIDR scientists has been the discovery of an enzyme in human gingiva which breaks down collagen fibers. This enzyme, collagenase, is being found both in human gingiva and skin. A major focus of current research is on the production, isolation, purification, and characterization of the enzyme. Related studies are being supported at the University of Oregon and at the University of Illinois to determine how collagenase relates to normal collagen synthesis and breakdown as well as to connective tissue disease.

Recent studies at the University of Southern California, using enzyme technics, confirm our understanding of the collagen molecule as composed of 3 polypeptide chains which form a rigid, rod-like structure. The structure is stabilized by a regular array of interchain hydrogen bonds together with steriochemical restrictives that are derived from the high content of pyrrolidine rings

(proline and hydroxyproline). This cable-like structure has great stability and tensile strength.

There is evidence, now, to support the hypothesis that the intramolecular cross-links contribute stability to the collagen structure. To ascertain this conclusion, rats with chemically induced lathyrism were studied. Characteristically, their periodontal tissues were constituted of fine, disoriented collagen fibers with marked reduction in the formation of long chain pair components. From these observations it would appear that the basic cause of periodontal disorders in experimentally induced lathyrism is an absence of intramolecular collagen cross-links. Further evidence that cross-linking proceeds at different rates and to different degrees in different tissues and animal species suggests an important maturation process which is required for proper functioning.

A number of etiological factors, including calculus and bacterial infection, have been associated with periodontal diseases. During FY 1969, the NIDR awarded a total of approximately $480,000 for projects aiming to identify the specific roles played by these factors. For example, studies at the University of Minnesota have confirmed earlier findings of Forsyth Dental Center investigators that bacteroides melaninogenicus is implicated in periodontal disease. This organism is not found in infant mouths, but begins to appear when the teeth erupt. Significantly, it is present in about 40% of children age 5 to 7 years; in more than 50% of children by age 9 years; and in nearly all children by age 13 years. In terms of concentration, the organism accounts for only about 2.6 percent of the total oral microflora in healthy mouths, whereas in patients with periodontal disease the percentage increases to as high at 7.8%.

There are several reasons for implicating melaninogenicus in periodontal disease. It ferments carbohydrates liberating certain types of acids, produces such tissue irritants as ammonia and hydrogen sulfide, and releases endotoxin and considerable amounts of collagenase.

In another area of study, the chromosome defect, trisomy 21 (mongolism), was found to be related to periodontal disease. Children with this hereditary disorder, cannot practice good oral hygiene and, accordingly, accumulate sufficient debris in their mouths to develop severe periodontal disease with considerable loss of bone. The bacteria in the mouths of 41 such children were compared with samplings from children institutionalized for cerebral palsy and from normal children having healthy gums. In the age range of 5 to 7 years, melaninogenicus was found in 63% of the trisomy-21 group; in 17% of the cerebral palsy group; and in 0% of the normal group. In the 9 to 10 year age range, 80% of the trisomy-21 children were infected whereas none of the normal children showed the microorganism. No cerebral palsy children were available in this age bracket for comparison.

Oral Neoplasms

Approximately 7,000 deaths are attributed each year to oral cancer. It is further estimated that approximately 22% of oral cancer patients are in need of some form of maxillo-facial prosthesis, and that even with proper surgical treatment, the complication of psychological factors associated with orofacial disfigurement, often compound the frequent difficulties in speaking, eating and breathing.

Present research efforts in oral cancer center mainly around the premalignant and malignant changes in the oral epithelium. About 90% of mouth-throat neoplasms are of the squamous or epidemoid variety. Premalignant changes in the epithelium are clinically diagnosed as leukoplakia, leukoderma, and oral whitespot. Recently, an NIDR supported long-term study at the University Hospital and the Royal Dental College in Copenhagen, Denmark, showed cancerous transformation in 4.4% of 248 patients with leukoplakia. A further finding of significance was that so-called "speckled" leukoplakia undergoes a greater degree of abnormal epithelial change than does any other clinical type of leukoplakia. The observation that seven out of the eleven patients who developed oral cancer had leukoplakia of the nodular type indicates the need for careful examination.

Studies recently begun at Tufts University School of Medicine and School of Dental Medicine are seeking to investigate all lesions classified clinically as leukoplakia. As part of the overall protocol, biopsies of hyperkeratosis, parakeratosis, acanthosis, dyskeratosis and carcinoma are being analyzed for enzyme, histochemical, and fine structure changes.

The NIDR is also supporting a limited number of basic animal studies in oral cancer. For example, at the University of Maryland, carcinoma of the hamster cheek pouch is being induced by topical application of 0.05% dimethyl, 1-2 benzanthracene, following which tumor development is observed for a 10-month period. Preliminary reports show that those animals subjected to ovariectomy have the greatest increase in tumor incidence. Somewhat lesser influences were related to estrogen administration and to norethynodrel plus ovariectomy.

Recent experiments at the University of Texas by NIDR supported grantees suggest that the marmoset is the animal of choice for the search for a human osteosarcoma virus. While earlier evidence has indicated that human osteosarcoma is caused by an infectious agent, the Texas researchers successfully induced osteosarcoma in two marmosets injected with the Rouse Sarcoma Virus Tumors were also produced when the virus was injected intramuscularly, subcutaneously, or into the liver of animals within their first 2 weeks of life. Forty-five percent of the marmosets surviving these procedures developed osteosarcomas, fibrosarcomas, rhabdomyosarcomas, or a mixture of fibro and rhabdomyosarcomas. In each instance, the type of tumor depended on the inoculation site. Thus, osteosarcomas appeared when the virus was injected into the jawbone, but there was no metastasis. On the other hand, marmosets with fibrosarcomas and rhabdomyosarcomas showed every evidence of metastasis.

Salivary Gland Disorders

The role of the salivary glands in regulating the oral environment is an important one. The normal integrity of the tissue or organ surfaces, both hard and soft, is dependent on a protective flow of salivary fluids. The microbial ecology of the oral cavity is undoubtedly influenced by the quantity and nature of salivary secretions. When the glands fail to function properly, rampant dental caries may develop even in elderly people whose teeth previously were resistant to dental decay.

Past and current support of research on the salivary glands shows a major attention to normal structure and function. In general, these relate to three objectives; 1) to clarify the biochemical events in the metabolism of amino acids by salivary gland cells, and determine the sites of transfer of amino acids and their metabolites into saliva. (Here the long-term goal would be to delineate fully the mechanisms of secretion of the organic compounds found in the saliva and to clarify the biological significance of these secretory components); 2) to delineate the mechanisms by which the electrolytic composition (chloride, sodium, bicarbonate) and electrical characteristics of resting and actively secreting salivary gland cells are maintained; and 3) to define the fole of the autonomic nervous system in the maintenance of normal structure and function.

Continuing studies at the University of Alabama concerned with the autonomic regulation of salivary gland function in man have measured salivary flow rate, and salivary levels of amylase, Na, K, and total protein. Among the findings of note are that the salivary stimulant, pilocarpine, gives much higher levels of amylase and total protein than does mecholyl or reflexly-mediated stimulation. These results appear promising for delineating the regulating factors of salivary function in man.

Chemical and ultrastructural studies, also at Alabama, are attempting to delineate the morphological aspects of transport in salivary glands. Pilot experiments indicate that the striated duct cells of the salivary glands comprise a specialized epithelium involved in fluid transport and that these cells might provide the morphological basis for transport in "quanta" by the salivary glands.

Similar in vivo studies dealing with electrolyte transport mechanism of the submaxillary glands are being conducted at Boston University. Here, findings indicate that although secretion is dependent upon Na^+ and K^+ activity at the acini lumen and vascular surfaces, concentration levels of ions in saliva depend on the resorptive activities of the luminal membrane of the ducts.

Investigators at the Forsyth Dental Center have demonstrated the presence of two antibacterial systems in human saliva which require thiocyanate for their activity. In the non-peroxidative system, bacterial inhibition is directly related to thiocyanate concentrations in saliva. Additional studies have found that thiocyanate levels in human parotid saliva vary inversely with rate of flow and follow a diurnal pattern, being lower during the sleeping hours. It is suggested that these fluctuations in thiocyanate concentration could account

for the observed variability in the activity of non-peroxidative antibacterial substances in saliva. Further studies are in progress to clarify the phenomenon of "thiocyanation" of susceptible bacteria. The question is whether an intermediate product is formed by the action of a salivary enzyme on the chemical chiocyanate prior to the inhibition of bacterial growth, or whether growth inhibition is the result of an "acceptor material" which is part of the cellular structure of the bacterium. A substance resembling the "acceptor material" has been isolated from bacterial cells.

In cases of human malnutrition the parotid glands are often observed to enlarge much beyond their normal size. Very little is known about the mechanism involved and at the University of Washington studies are being conducted on animals to seek out the causative factors involved in the observed phenomenon. The results of these studies to date have shown that in adult male rats, reared on a low intake of food, hyperthrophy of the gland between 1-12 days is mainly due to reduced expulsion of secretory fluids and hence, the storage of secretory components in the acinar cells produces enlargement of the gland. During the second 12 days the gland adjusts to the reduced amount of food intake.

Basic studies which deal with secretory cell recovery after prolonged obstruction, have an important bearing on questions relating to the biochemistry of acinar and ductal secretions as well as on the relationship between functional competence and differentiation of specialized cells. The studies carried on at the University of Washington have led to a better understanding of cellular changes in clinical problems of obstructive adentitis in patients with various diseases of the salivary glands. For example, from electron microscopic study of human parotid mucoepidermoid carcinoma it is possible to differentiate anaplastic from dysplastic cells in a pleomorphic cell population. It was recognized that some of the pleomorphism in the tumor was identical to changes in the experimental rat glands, and that some of the cellular alterations were due to ductal obstruction rather than neoplastic differentiation. These findings could result in a reconsideration of the criteria usually applied to the classification of salivary gland tumors.

Oral Ulcerations

Several distinct diseases are either manifested as or accompanied by ulcerative lesions which affect the oral mucous membranes. Ulcerations of the oral tissues occur in such diseases as aphthous stomatitis, periadenitis mucosa necrotica recurrens, herpes labialis, Behcet's Syndrome, erythema multiforme, lichen planus, pemphigus vulgaris, and pemphigoid. These diseases and the oral ulcerations which they cause vary in duration and severity. Pemphigus vulgaris still results in a very high mortality rate (40-50 percent) even though treatment with corticosteroids has been successful in some cases. In Behcet's Syndrome, periadenitis, and some cases of aphthous stomatitis the oral mucosa is the site of continuously recurring outbreaks of multiple ulcerations which seriously debilitate and incapacitate the affected individual. In other instances, lesions may be limited to occasional episodes of painful but relatively innocuous aphthae and blisters.

With the exception of herpes labialis, which is considered to result from a reactivation of herpes simplex virus infection, the etiologies of these other diseases which cause intra-oral ulcerations are unknown. In many instances a definitive diagnosis is difficult to establish. Except for the use of systemic corticosteroids in pemphigus and erythema multiforme, treatment is generally ineffective.

Grantees at the University of Oregon are currently investigating cases of lichen planus by histochemical techniques. It is postulated that the lymphoid elements are responsible for both the lytic and the coagulative changes seen in oral epithelium. It is hypothesized that these changes represent a biphasic form of immunologic disease precipitated by stress and/or some alteration of an antigenic component of the epithelial cells located in the basal cell area.

Another example of research in the area of oral ulcerations is that being carried out by grantees at the Philadelphia General Hospital. Here, investigations include epidemiological studies or recurrent aphthous stomatitis and herpes labialis, immunoglobulin levels in persons with recurrent intra-oral herpes simplex infections, the role of PPLO and L-forms in recurrent aphthous ulcerations, and the effects of treatment of oral ulcerations with several tetracyclines and corticosteroids.

It is obvious that knowledge from many scientific disciplines must be brought to bear to solve problems created by oral diseases. However, advancement has been slow as a consequence of the shortage of trained investigators and limited availability of research funds. Nevertheless, current research covers a rather broad range of activity which includes immunological mechanisms and hypersensitivity reactions; the roles of bacteria, viruses, and emotional stress; and clinical trials of the therapeutic value of various antibiotics and steroid compounds.

Oral-Facial Growth and Development Program

The Oral-Facial Growth and Development Program encompasses three major research areas: oral-facial malformations and defects; malocclusion; and functional disorders of the oral-facial complex. Support, through grants and contracts, of research pertaining to etiology, prevention, correction, and other rehabilitative aspects of oral-facial malformations, especially cleft lip and cleft palate, continues to be of high program relevance. Also in order to alleviate the lack of sufficient research manpower, the Oral-Facial Growth and Development Program administers the support mechanisms of research training grants, fellowships, and career development awards within its scope.

Scientific Evaluation of Specific Research Areas

Under the chairmanship of Dr. F. Clarke Fraser, McGill University, who is a member of the Oral-Facial Growth and Development Program ad hoc advisory panel, a workshop on the genetics of the lip and palate was convened on October 3 and 4, 1968. Thirteen leading scientists participated in the two-day work sessions designed to evaluate past and current research in this field, and to advise on the directions of future investigations. One significant accomplishment of the workshop was agreement of its participants to pool their research data so that a larger sample of meaningful genetic information could be made available to the literature.

The proceedings of a "Symposium on Bone Growth as Revealed by In Vivo Markers" which was held on April 26, 1967, at the 36th Annual Meeting of the American Association of Physical Anthropologists, Chapel Hill, North Carolina, was recently published in the American Journal of Anthropology, Volume 29, Pages 155-310, 1968. This scientific evaluation was supported under research grant DE 02604 (Principal Investigator: Melvyn J. Baer, University of Michigan).

In another area of interest, the Oral-Facial Growth and Development Program is planning a workshop on the "Food and Nutrition of Nonhuman Primates." Under the chairmanship of Dr. Robert S. Harris, Professor of Nutritional Biochemistry, Massachusetts Institute of Technology, and the co-chairmanship of Dr. Thomas B. Clarkson, Professor and Head, Department of Laboratory and Animal Medicine, Bowman Gray School of Medicine, Wake Forest University, this meeting will seek to evaluate the entire subject of primate diets.

The field of speech continues to receive priority attention. For example, a contract proposal from the American Speech and Hearing Association is designed to undertake an in-depth assessment of needs and accomplishments as a basis for the setting of program goals and priorities in Communicative Science research.

During FY 1969, our evaluation of the etiological aspects of cleft lip/cleft palate, which was published in Science, Volume 158, Pages 1603-1606, 1967, was reprinted in two other journals; Cleft Palate (Volume 5, Pages 187-194, July 1968) and Teratology (Volume 1, Number 3, Pages 353-358, August 1968). In addition,

summary accounts appeared in such publications as Rehabilitation Literature, National Easter Seal Society for Crippled Children and Adults (Volume 29, Number 11, Page 342, November 1968).

Cleft Palate Programs and Projects

A survey, initiated this year, of U.S. institutions that provide cleft lip/cleft palate services is expected to be completed soon, and made available for publication. This work involves a joint effort by Dr. Charles Kram, Chief Psychologist, Jackson Memorial Hospital, Miami, Florida, and Mrs. Helen Riches, Reports and Analysis Section, NIDR.

Because of their surgical, dental, speech, and psychological aspects, problems arising in cleft patients require a team approach for successful management. The National Institute of Dental Research supports five cleft palate centers in which treatment and research are conducted in an interdisciplinary manner.

At the University of Pittsburgh and at the Universite Libre de Bruxelles, tissue culture techniques have been utilized to throw light on the normal fusion process of cells in the palatal tissues in A/Jax mice. These investigators have shown that palatal shelves, whether from cleft or normal embryos, will fuse when they are placed in proximity to each other. Accordingly, the search continues for extraneous factors that will prevent fusion.

At the University of Michigan investigators are making histochemical and ultrastructural analyses of normal developing mice and rat palatal shelf tissues at stages shortly before and during fusion. Technics of study include in vitro culturing of eyelids and eyelid-palatal shelf combinations, as well as palatal shelves with and without the tongue interposed in its early embryonic position. To date, some progress has been made on the analysis of embryonic movements associated with palatal closure.

NIDR grantees at the University of Pittsburgh who had previously found more abnormally shaped teeth in patients with clefts of the lip and/or palate than in normal individuals have reported a high incidence of such defects in mental retardates. These investigators reason that teeth are more vulnerable to teratogens than most other organs because they begin to differentiate later and undergo development for much longer periods. They also suggested that tooth defects may indicate the time that genetic factors or an environmental insult cause correlative interferences with mental development.

More recent reports show that monkeys also have spontaneous clefts and therefore may be useful for cleft palate research. NIDR grantees at the University of Pittsburgh made the first histological study of a cleft palate in a marmoset fetus. Two other cases are mentioned in the literature. In addition, in February 1969, a 2-month old male rhesus with a cleft lip and cleft palate, born at the Hazelton Laboratories, Falls Church, Virginia, was brought to staff's attention. Since the mother of this malformed monkey came from a control group and had no drug history, arrangements were made for this specimen to be shipped to the Regional Primate Center, University of Washington, Seattle, Washington. NIDR-supported scientists at the Primate Center will

rear this animal to maturity for breeding and subsequent studies on the heritability of this malformation.

Since hearing is essential for adequate speech development, the prevention of even slight deafness in early years, when clefted children have ear infections most frequently, is very important. At Duke University surgeons report that removing adenoids at the time of palatal surgery prevents much of the expected middle ear inflamation.

Although surgery is often the key to successful management of clefted patients, diagnostic technics are needed to help the cleft palate team decide when to operate or when an obturator appliance would suffice. NIDR grantees are following the progress of patients on a longitudinal basis so that the criteria for selection of the most appropriate technics can be determined. In one study at Duke University, a five-year appraisal has been made of 25 infants who had bone grafted into the clefts. Preliminary reports indicate that although the grafts were accepted by the body, new tissue growth was not entirely satisfactory.

Studies of the levels of air pressure in the nose and mouth, helpful in predicting the capacity for good speech in cleft palate patients, are receiving particular attention at the Eastman Dental Center in Rochester, New York. Highly sensitive equipment, including x-ray motion pictures synchronized with a high fidelity sound recorder and a photographic recording of an oscillograph, is used to pick up on separate channels the air pressure, air flow, and air volume in the mouth and in the nose. These records are then analyzed to evaluate the sequence of certain sounds, as well as to help determine good speech capacity.

An important aspect of speech research is the study of the muscles involved. Scientists at the Haskins Laboratories in New York have recorded normal muscular patterns in speech as a basis for comparison with muscle reactions in subjects with impaired speech. These studies have led to the finding that production of a particular sound (phoneme) used in speech often requires more than one pattern of muscular motion.

Some insight into the role of speech codes has been provided by efforts of scientists to devise sound alphabets suitable for converting print into sound to help the blind read. The fact that, despite 50 years of experimentation, sound alphabets have achieved only about a tenth of the speech and information that comes easily through speech suggests that the only way the slow machinery of the ear can handle many signals rapidly is through code. Investigators at the Haskins Laboratories consider that most encoding takes place below the level of consciousness and before the neuromotor command is given to the articulatory muscles. This view is borne out by the fact that electromyography shows that some of the muscle action is always the same for making each sound, whereas the spectrographic pictures of sounds perceived as the same actually differ. Since there is no single way that any sound is perceived, some sort of "decoder" identifies incoming sounds by referring them to the commands for producing them by moving muscles.

This year a study of communication skills conducted at the University of Pittsburgh has thrown new light on the puzzling passiveness and lack of creativity of many cleft palate patients. The research shows that afflicted children are more likely to be deficient in motor responses than in vocal ones. Even normal speakers among them use few gestures. When hearing is normal, clefted children have as good an auditory memory and understanding of words as others. However, their ability to do something in response to visual or auditory stimuli is below normal. For example, clefted children find it more difficult to select similar objects from a group of pictures; to pantomime the use of an object; to duplicate, from a pile of pictures, a set of pictures previously shown and removed; or even to repeat a series of numbers. Tests of these communication skills show no improvement with age. Rather, they retrogress.

Scientists at the University of Pittsburgh have found that children who couple bad temper with speech that is not nasal but full of articulation errors need psychological rather than physical help for speech improvement. One hundred seventy clefted children, aged 3 to 10 years, were grouped according to type of articulation in a study of psychological problems. All the subjects had normal hearing and intelligence although the I.Q. of poorer speakers was slightly lower.

Speech performance was correlated with the absence or presence of 29 behavioral problems as rated by the children's mothers. The good speakers had fewer behavioral problems although they showed a greater tendency to fight than others involved in the study. The scientists believe that children who can control closure of the gap between palate and throat often fail to speak well because they need psychological help to manage their emotions.

Patients and parents were asked to evaluate the effects of surgery on speech and social adjustment in a psychological study at the Eastman Dental Center. Parents and patients both felt that speech had improved more than professional tests showed. Both groups also felt that social adjustment had improved, although the parents appeared to be more impressed with this factor than did the patients.

A new program-project, "Center for Craniofacial Anomalies," was initiated at the University of Illinois. The primary mission of the Center is to conduct longitudinal studies on children with congenital and developmental anomalies of the craniofacial complex. This interdisciplinary effort will be concerned with congenital or acquired organic defects of the craniofacial structures in children and adults which are chronic and sufficiently disabling to present an intertwined medical, dental, emotional, social, educational, and/or vocational problem requiring prolonged supervision for optimal rehabilitation. The primary method of study is to record descriptive data of the parameters of growth and function over time on these subjects. Description through dental casts and cephalometric x-rays is supplemented by case histories, clinical medical records, tape recordings for speech analyses, audiology testing, and related psychological, educational, and clinical records. The approach of the Center team is to collect and later review and study the longitudinal material on hand in order to generate descriptions and questions regarding

possible effects on growth, and specific effects of surgical and dental manipulation over time on various types of disorders.

Another new NIDR program-project grant is supporting a study of "Oral-Facial Growth and Development in Primates" at the University of Washington. The proposed objectives of this project include the development of a primate center for the production of Macaca nemistrina (pigtail monkey) in sufficient quantities for longitudinal studies of growth and development of oral tissues. With respect to dentition and the facial maxillary region, the monkey is more similar to man than other non-primate laboratory animals. However, dental research on monkeys has been largely confined to late adolescent and young adult animals. The many problems relating to dental and facio-maxillary development, including teratology, have not been substantially investigated because of the difficulties in breeding monkeys in captivity. The choice of a monkey species eminently suited to breeding, the experience gained by the principal investigator over the past ten years with this species, and the extensive space and facilities available at the Primate Center make it extremely worthwhile to stage this large-scale breeding program in support of dental research. The specific areas of research which are being pursued include 1) oral-facial development, 2) cleft lip and palate, 3) dental development and genetics, 4) pediatric endodontics, and 5) developmental changes in biochemistry of saliva and salivary glands.

Malocclusion

Studies in the area of orthodontics supported by the Oral-Facial Growth and Development Program have been concerned with the etiology, prevention, and treatment of malocclusion.

Under NIDR support, investigators at the University of California at San Francisco have established a primate laboratory where the rhesus monkey is being utilized as a valuable experimental model in studies on the etiology of malocclusion. In a biologic framework it may be stated that if specific neuromuscular patterns produce malocclusions in man, there may be analogous factors producing similar deviations in other primates which present similar morphologic structures and functional behavior. By either reducing or exaggerating specific neuromuscular processes in common functional patterns in primates and correlating the resultant deviant orofacial morphogenesis, the investigators feel we will be much closer to understanding the etiology of malocclusion in man. Attention will be focused on the role played by neuromuscular function in crowding and spacing of teeth, jaw growth, and Class II malocclusion. Recent pilot studies have supported the responsive role the dental arches play in relation to alterations of neuromuscular function. The function and posture of the tongue in the rhesus, altered either through a prosthesis or surgical intervention, were found to have a direct influence on the spacing, crowding, and occlusion of the teeth. With continued and expanded experimentation it is hoped that we shall approach an understanding of the etiology of malocclusion.

Exhaustive collection and analysis of physical data on human subjects have been the basis of two separate studies presently continuing at Northwestern

University and State University of New York at Buffalo. The Northwestern study involves the collection of study casts, cephalometric and hard-wrist radiographs, facial and intra-oral photographs, and height and weight recordings in an attempt to relate dento-facial development to body growth. At the present time a cephalometric analysis of Class I occlusion subjects in different chronological and skeletal age categories is progressing in order to assess specific variations related to age and sex.

The State University of New York (Buffalo) investigation is a longitudinal analysis of the dental, facial, and general body growth changes manifested in increments, velocities, and patterns during the midchildhood and adolescent periods in human twins. The goals of the study are to determine the nature of the relationships between the attributes of physical growth and hereditary components of developing maturational systems, such as the dentition and the skeleton. Increased accuracy in the assessment of growth potential and timing of growth accelerations would be of tremendous value to the orthodontist in treating dento-facial abnormalities. The investigators have recently established a high correlation between lower second premolar traits and serological traits in monozygotic twins. These findings provide impetus for establishing genetically determined traits of other teeth to determine the usefulness of dental traits combinations in establishing twin zygosity.

Studies in interceptive orthodontic procedures are being performed in Navajo Indian children at the PHS Indian Hospital, Gallup, New Mexico, in hopes of determining limited orthodontic procedures which would be effective in reducing the occurrence of handicapping malocclusions. Serial extraction therapy is being analyzed using cephalometric coordinates. Such analyses will determine mean cephalometric measurements of various ages among the Indian population as well as the effect of serial extraction on these measurements. A recent publication derived in part from these studies is a manual of "Preventive Orthodontics and Limited Treatment Procedures," published by the U.S. Department of Health, Education, and Welfare, PHS, Division of Indian Health.

Functional Disorders

Detailed studies of both the qualitative and quantitative aspects of mandibular motion are in progress. Investigators at Marquette University have developed an analog-computer system of recording and processing jaw motion data of patients performing various masticatory movements. The data analyzed indicates very little possibility of a true hinge axis motion. It is presumed that as additional information is gained the diagnosis and treatment of patients with abnormal masticatory function will be improved.

A study undertaken at the University of Pennsylvania is attempting to determine the electrophysiologic effect of Golgi tendon organ stimulation on cyclic jaw movement. Recent efforts have been directed towards discriminating the geneses and characteristics of the electromyographic pause observed in masticatory muscles. It is becoming increasingly apparent that understanding the nature of the mechanism of the silent period is intimately related to the study of Golgi tendon organs, and future efforts will be continued in that direction.

This project should provide some clearer insight into involuntary movements of the jaws due to muscle flexion and extension operating in a balanced fashion.

At the University of Illinois Medical Center in Chicago, investigators are testing a theory that tension-relieving oral habits related to emotional problems can lead to severe, yet difficult-to-diagnose facial pains of temporomandibular joint dysfunction. The investigators plan extensive clinical, physiological, and psychological examinations to aid in the diagnosis and treatment of this condition.

Contracts

As an extension of the study conducted under contract No. PH 43-68-1317 (Principal Investigator: Dr. William S. Laughlin - "Dento-Cranial Studies of a Genetic Isolate of Eskimos"), contract No. NIH 69-2054 ($2,900) was awarded to the University of Michigan (Dr. Stanley Garn). This contract is entitled "A Follow-up Study on the Dento-Cranial Studies of a Genetic Isolate of Eskimos" (2-26-69 - 8-25-69). In this extension study, Dr. Makoto Matsumoto and Dr. Stanley Garn are analyzing the data accumulated during the summer of 1968 utilizing the unique Michigan facilities which include dial-reading, print-out and card punching measuring devices with 0.1 mm. readout, banks of radiographic illuminators, two IBM printing punches, and terminals to the University of Michigan computing center, as well as desk type print-out electronic calculators.

The thickness and size of component bones, the contribution of sinus enlargement to the development of the face and head, the growth and possible loss of cortical thickness, as well as specifics to tooth formation and movement of bone loss, will be ascertained. A specially devised matched pair program will match each Wainwright Eskimo individual with an American white of matched age and sex.

Research Training

The Oral-Facial Growth and Development Program will be supporting 31 research training grants at a level of $1,609,000 on June 30, 1969. This includes three new training grants which should help to meet the need for geneticists, anatomists, and pharmacologists with specific orientation to dental-related research.

A renewal training grant proposal submitted by the American Speech and Hearing Association to continue the activities of the Joint Committee on Dentistry and Speech Pathology was approved at the March National Advisory Dental Research Council meeting. This activity will continue to be supported for three more years, and it is expected that fruitful interactions between the American Speech and Hearing Association and the American Association of Dental Schools will continue.

In this fiscal year, 20 fellowships were supported at a level of $199,000. This reflects a significant increase since the number of fellowships awarded doubles the number of fellowships funded in FY 1968.

Twelve career development awards were funded at a level of $233,000.

Use of CO-STEP

A first year dental student from the University of Maryland, a reserve commissioned corps officer (COSTEP), was assigned for duty to the Oral-Facial Growth and Development Program for three summer months. This is the first time that a co-step was assigned to the Extramural Programs. This young officer assisted in the analysis of the program area as well as participated in the preparation of the Cleft Palate Service Directory. This effort not only served as a useful auxiliary support for the program, but fostered a worthwhile scientific experience for the participant.

Materials Science and Special Clinical Studies Program

The ultimate objectives of the Materials Science and Special Clinical Studies program are to increase the availability and efficacy of dental care through research and development of new and improved materials and methods of treatment. Under this mission support is provided by contracts, research grants and training grants to various projects of both basic and applied research nature. These projects involve a multitude of physical and biological science disciplines, including engineering, physics, crystallography, metallurgy, polymer chemistry, radiology, pharmacology, oral surgery, and immunology. Selected descriptions of findings obtained in projects supported during the current year are included in the text which follows.

A study at Indiana University has shown that cement fillings are and remain more acidic (pH about 5.5) than previously thought and that components of the cement can penetrate considerable thicknesses of dentin. As a result, the investigators have recommended that bases of zinc oxide eugenol or calcium hydroxide, but not copal varnishes, be used as a lining to protect the pulp from the acidic, silicate cement fillings. In vivo studies in monkey teeth, as well as examinations of extracted human teeth show that these bases stop acid leakage, yet allow fluoride to seep out from the filling to the surrounding tooth where it helps stop further decay. Although a copal varnish also was shown to protect the pulp, it should not be used because it blocks fluoride leakage. However, varnishes are useful with amalgam fillings because they eliminate micro-leakage which can cause sensitivity, pulpal damage, and tooth discoloration. To measure the acid penetration, silicate cements were tagged with radioactive phosphoric acid and counts were made on tooth sections. When a base was placed under the filling, the average measure of radioactivity obtained in the section closest to the cavity floor was less than four percent of the average count obtained when no base or varnish was used. Both bases, either 0.1 or 0.2 mm thick, were equally effective. In a related study at the University of Indiana the effect of fluoride containing zinc oxide-eugenol on the solubility of enamel was examined. Sodium and stannous fluoride were added in quantities of 2%, 5%, 10% and 15% to the powder component of a commercial zinc oxide and eugenol cement. All of the tests showed a reduction in enamel solubility with the maximum reduction being achieved in less than one week of contact. The tooth structure itself showed a corresponding increase in fluoride content.

At the University of Alabama an extensive study of the effects of a number of variables on compressive and tensile strengths of silicate cements has been conducted. Ten different commercial silicate cements were thoroughly studied, and through the use of computer technics the data was processed and related to the strength properties. A significant variable was the powder-liquid ratio. Statistical analysis indicated that a 0.1 increase in powder-liquid ratio would increase compressive strength by about 1500 lbs. per square inch and tensile strength by 100 lbs. per square inch. In a study at the National Bureau of Standards plastic reinforced EBA cements were tested as restorative materials. In this study polymeric powders were incorporated into alumina-

reinforced zinc oxide eugenol-o-ethoxybenzoic acid cements. Such formulations resulted in a filling material of suitable properties for use as an improved temporary filling. A limited number of clinical restorations have remained serviceable for 8 month periods without showing signs of wear, even though some of these restorations were subjected to heavy occlusal stresses. In another study at the National Bureau of Standards certain surface active compounds are being examined to see how well they serve as primers to improve adhesion bonding between polymerizing formulations and tooth surfaces. In some of these studies such agents have significantly increased the average tensile bond strength. Workers at the Bureau of Standards have also investigated the amines which are used to accelerate polymerization. Several compounds of this type are being studied in relationship to discoloration, toxicity and other properties of the resulting polymers. The setting times and color stability of various specimens were found to depend upon the size and nature of the substituent groups on the amine.

In animal and human studies at the State University of New York at Buffalo evaluations were made of a cyanoacrylate cavity liner. Pulpal reactions to the material in monkeys was less than that obtained when calcium hydroxide was used. The sealing properties were considered promising for human use. At the Polytechnic Institute of Brooklyn an extensive metallurgical study has been made of gold-nickel alloys containing between 30% and 70% nickel. The properties of this type of alloy have been improved by special treatment. Thus, crowns cast from the alloy have been observed clinically over a period of several years and found to be satisfactory. Furthermore, specimens implanted subcutaneously in rats for one month have shown essentially no corrosion.

At the University of Utah investigations of bond rupture in teeth and dental materials using the technic of electron paramagnetic resonance have been performed. In studies of tooth decay by this technic it was evident that molecular rupture of structural bonds extended approximately 0.5 millimeter beyond the point where carious discoloration ends. In another study at this same institution teeth were exposed to thermal cycling tests in which the temperature of the ambient fluids ranged from 40° to 150° F., a temperature range thought to simulate that which actually occurs in the human mouth. After less than 1,000 cycles, significant cracking of tooth structure appeared. Thermal fatigue studies of this type are also being carried on in teeth with fillings.

Ongoing studies at Brown University of the development of dental implants have demonstrated in the past that it is possible to successfully implant a plastic tooth replica in the socket of a recently extracted tooth. Such implants have now remained functional in baboons for as long as eight years. One of the findings claimed in this study is that periodontal fibers grow into natural voids in the plastic tooth and thus increase tooth stability. When large holes one or two millimeters in diameter have been created in the plastic tooth roots it was found that this space becomes filled not only with fibers but by a bridge of natural bone which helps to anchor the tooth. In present attempts to study the effect of fibrous ingrowth into plastic implants, foaming agents are being used to increase the porosity of the tooth root. In another portion

of this same study natural tooth replants are being coated with plastic before replantation. This procedure is said to prevent the root resorption which has been reported in other studies in which the tooth root has not been coated with plastic.

In tooth transplant studies at Harvard University the reattachment process of mature adjacent autologous and allogenic tooth transplants was studied for additional evidence on the etiology of allograft tooth resorption. One maxillary central incisor autograft and one allograft were performed side by side in each of ten rhesus monkeys. Whereas the autograft exhibited near normal reattachment by four weeks, the allograft never did. The four week allograft showed extensive lymphoid infiltration of the periodontal membrane with progressive widening thereafter. A definitive cementoblastic layer and functional fiber orientation, present in the autograft, never developed in the allograft. The long term allograft was characterized by progressive vertical bone loss, ankylosis, and extensive generalized inflammatory root resorption. These studies suggest that donor periodontal ligament, although critical to autograft success, appears to contribute to allograft failure. The rejection of allogenic tooth transplant was somewhat inhibited by pretreating the transplant teeth with proteolytic enzymes.

In a continuing clinical study at Eastman Dental Center attempts have been made to prevent pit and fissure caries by protecting these susceptible areas with plastic coatings. Using a new formulation that can be cured in situ instantaneously by means of ultraviolet light, it has been possible to successfully retain the coatings on teeth for as long as 13 months. The coatings show no signs of wear and appear to competently seal off the susceptible areas. The effects of this treatment on the incidence of caries is presently being evaluated.

THE REPORT OF THE DIRECTOR OF INTRAMURAL RESEARCH

THE NATIONAL INSTITUTE OF DENTAL RESEARCH

July 1, 1968 - June 30, 1969

(Report not ready at time of printing.)

Report of the Environmental Mechanisms Section
National Institute of Dental Research
Summary Statement

The Environmental Mechanisms Section was established to fill a need within the Institute for investigations into systems of controlled and uncontrolled interaction between a cell or group of cells and its environment, as related to oral biology.

One level on which cells 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 slime mold, Dictyostelium discoideum.

A less organized bio-system under study in the Environmental Mechanisms 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; their minimal nutritional requirements; 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 year just passed are described in the following paragraphs.

In investigating the biochemical factors initiating and controlling morphogenetic development of Dictyostelium discoideum, the main efforts are concerned with those parameters affecting the initiation of differentiation by chemotactic aggregation as well as subsequent control of the rate of morphogenesis. Based on the work to date, it seems likely the ribose mononucleotides, formed by degradation of endogenous RNA, play a central role in the control of differentiation. As the 3',5'-cyclic monophosphates they provide the directional information for differentiation. The degradation of the 3',5'-monophosphate by the non-specific extracellular phosphodiesterase results in the formation of the compounds capable of accelerating the morphogenesis. The mechanisms by which these effects are mediated are currently under investigation.

The multifaceted study of the environmental control of the cellular physiology of the caries-conducive streptococci has been quite fruitful. The direct demonstration of polyphosphate formation in high yield by non-growing cells calls attention to the concept that plaque-forming streptococci may represent a continuous sink for tooth phosphate as long as carbohydrate is present in the cell's environment. The finding that plaque-forming streptococci grow slowly and linearly in the presence of sucrose suggests that the study of the physiology of slow growing or non-growing cells is more relevant to understanding oral pathosis than is study of exponentially growing cultures. These last findings have important implications for any model to be constructed in relation to the dynamics of caries production by the streptococci under study. An understanding of these dynamics will be greatly aided by our increased capabilities in the area of mathematical models of bacterial growth. Such models will provide useful guidance in the analysis of data taken in the detailed study of the growth of caries conducive streptococci.

Without an understanding of the overall details of the nutritional requirements of these organisms, performance of the foregoing experiments and their interpretation are questionable. Since it often takes 1-2 man years to elucidate the nutrition of strain of fastidious bacteria such as these, the design of a computer-controlled machine to perform this task has commenced.

In this vein also, it should be noted that many of the above studies have been substantially aided, and in some instances even made feasible by the application of computer technology and systems analysis. New data acquisition and reduction methods have had to be devised as required. Currently, time-sharing facilities have been acquired and are being used in support of ongoing investigations. In addition, a Honeywell 516 computer system for on-site use within NIDR has been selected for installation in the near future. Software and hardware development, as well as site preparation in anticipation of this expanded capability for ADP has accounted for much effort this year.

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

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

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

Previous Serial Number: NIDR-3

Principal Investigator: Dr. M.I. Krichevsky

Other Investigators: Dr. B.M. Chassy

Cooperating Units: None

Man Years:

Total:	1 3/4
Professional:	1/2
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. Since the first observable event in the morphogenesis is aggregation of scattered single cells, the chemotaxis is also being studied.

Methods Employed:

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

Major Findings:

Ribose mononucleotides accelerate morphogenesis in Dictyostelium discoideum at 10^{-4} M and above. Equimolar purines, pyrimidines, ribose, phosphate, ribose-5-phosphate and nucleosides either slowed morphogenesis or had no effect; 5'-GMP, 5'-UMP and 5'-CMP were about as effective as AMP. The comparable 2',3'-cyclic mononucleotides and 2' or 3'-mononucleotides were also effective. Thus the position of attachment of the phosphate to the ribose moiety is not critical.

However, 3',5'-cyclic ribose mononucleotides act as chemotactic messengers in addition to showing the ability to accelerate morphogenesis. The dibutyryl analogue of 3',5'-AMP accelerated morphogenesis without acting as a chemotactic agent.

Significance to Dental Research:

The phenomena of nongenetic functional changes in cells, as typified by embryological differentiation, microbial spore formation, induced enzyme formation, cancerous de-differentiation and aging, 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.

The biosynthesis of the cyclic mononucleotides will be determined and correlated with steroid biosynthesis and growth.

Part B

Publications:

1. Krichevsky, M.I., Love, L.L. and Chassy, B.M.: Acceleration of morphogenesis in Dictyostelium discoideum by exogenous mononucleotides. J. Gen. Microbiol. in press.

Serial No. NIDR-2 (68)
1. Office of the Director of Intramural Research
2. Environmental Mechanisms Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Metabolic Control of Morphogenesis and Differentiation in the Cellular Slime Mold, Dictyostelium discoideum.

Previous Serial Number: None

Principal Investigator: Dr. B.M. Chassy

Other Investigators: None

Cooperating Units: None

Man Years:

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

Project Description:

Objectives:

The overall objective of this project is to understand the factors controlling aggregation and morphogenesis of the myxamoebae Dictyostelium discoideum.

The specific goals are 1) to purify and characterize the extracellular phosphodiesterase secreted by this organism, 2) to assess the enzyme's role in mediating differentiation and 3) to determine if control of enzyme activity operates as part of a feedback system governing morphogenesis.

Methods Employed:

Standard enzyme handling techniques (ammonium sulfate fractionation, heat treatment, and DEAE-cellulose chromatography) have been employed. The enzyme has been assayed by a new procedure coupling phosphodiesterase activity directly to pyridine nucleotide oxidation. (ATP, adenylate kinase, pyruvate kinase, PEP, lactic dehydrogenase and NADPH in excess are used). A chemical synthesis

of commercially unavailable cyclic IMP was carried out.

In vivo experiments involve placing amoebae on washed agar plates impregnated with various compounds or the enzyme and watching the fate of differentiation. Photographic procedures have proven useful for recording differentiation with time.

Major Findings:

1. The cyclic nucleotide phosphodiesterase can be readily purified; although polyacrylamide gel electrophoresis shows it is not yet a pure protein.

2. The cyclic nucleotide phosphodiesterase is not specific for cyclic AMP, as previously reported. TMP-, UMP-, CMP-, and to a lesser degree GMP-, and IMP-cyclic phosphates are substrates. This is a particularly significant finding since these compounds are also "acrasins", or chemotactic agents, for the cellular slime molds.

3. The phosphodiesterase functions as the long-postulated "anti-acrasin" factor. That is, it mediates differentiation. High concentrations of enzyme can block aggregation by destroying acrasin, while lower concentrations would facilitate aggregation by setting up concentration gradients and providing a source of 5'-mononucleotides which are stimulatory to differentiation.

Significance to Dental Research:

A clear understanding of chemotaxis, differentiation and morphogenesis in a simple model system such as the cellular slime molds is not only of interest to general biology, but may be of direct significance to several dental problems. In particular, the processes involved in growth and differentiation of oral tissue, anomalous differentiation or phagocytic response to bacterial challenge, may all be better understood with a fundamental knowledge of chemotaxis, differentiation and morphogenesis in the cellular slime molds.

Proposed Course of Study:

1. To prepare homogeneous enzyme preparations.

2. To determine if "feedback" or "regulatory" phenomena operate with this enzyme.

3. To establish the role of the enzyme in the life cycle and as a component of the effects observed in vivo under certain

physiological conditions (e.g. the addition of steroid biosynthesis inhibitors, 5'-mononucleotides, histidine, etc.).

Part B

Publications:

1. Krichevsky, M.I., Love, L.L. and Chassy, B.M.: Acceleration of morphogenesis Dictyostelium discoideum by exogenous mononucleotides. J. Gen. Microbiology. in press.

Serial No. NIDR-3 (68)
1. Office of the Director of Intramural Research
2. Environmental Mechanisms Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Use of Specifically Tritiated Compounds in the Investigation of Enzyme Mechanisms

Previous Serial Number: None

Principal Investigator: Dr. B.M. Chassy

Other Investigators: None

Cooperating Units: None

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

Project Description:

Objectives:

The overall goal of this project is to use specifically tritiated ribose derivitives as a tool in the determination of certain enzyme mechanisms. Three distinct types of enzymes are to be studied: ribonucleotide reductases, which provide monomers for DNA biosynthesis; B_{12}-coenzyme (dimethyl benzimidazole deoxyadenosyl cobamide coenzyme) dependent enzymes; and pentose metabolism enzymes. Chemical and enzymatic synthesis of the substrates for these enzymes bearing tritium (^3H) in a specific position is the immediate objective.

Methods Employed:

Standard radioisotope handling for chemistry and enzymatic synthesis was employed. These involve paper, thin layer and column chromatographic procedures.

Major Findings:

This newly initiated project has resulted in the synthesis, via chemical and enzymatic methods, of ribose-5-phosphates with high radiochemical purity and specific activity. The specific labels are in the 3-position and the 4-position. These compounds have not been previously reported. Methodology for synthesis of ribose-5-phosphate-2-T, and ATP and B_{12}-coenzyme bearing each of these three ribose residues has been established.

Significance to Dental Research:

To the extent that these fundamental studies on detailed enzyme mechanism shed light on metabolic processes and physiological control, they contribute to our understanding of oral tissue and the flora it hosts. In particular, neither oral tissue nor its bacterial inhabitants has been assessed with respect to the role of Vitamin-B_{12}-coenzyme in their metabolism. In addition, the place of pentose metabolism in cariogenic streptococci is, as yet, unstudied.

Proposed Course of Study:

1. To synthesize ribose-5-phosphate-2T, ATP-2'-,3'-, and 4'-T, as well as B_{12}-coenzyme-2'-,3'-and 4'-T.

2. To look for hydride elimination, hydride shifts and stereospecificity in the ribonucleotide reducatases from Lactobacillus leichmanii and Escherichia coli.

3. To assess the role of the 4'-proton in B_{12}-coenzyme dependent reactions such as: diol dehydrase, ethanolamine deaminase, and ribonucleotide reductase.

4. To investigate the hydride losses and shifts in the pentose-phosphate isomerases and epimerases of the lactic acid bacteria.

5. This project will be spread out over a number of years. The general procedures involved are to be integrated into our major studies on growth and differentiation as well as sucrose metabolism in cariogenic streptococci. The handling of data of amassed in this kind of study will be greatly facilitated by the use of an on-line computing system.

Part B not included

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

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

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

Previous Serial Number: Same

Principal Investigator: Dr. J.M. Tanzer

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	3/4
Professional:	1/2
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 multisurface caries.

The immediate goal of the present phase of study is to establish whether these plaque-forming streptococci are capable of forming polyphosphates.

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 ^{32}P monitored. These techniques were previously detailed (Tanzer, Annual Report 1966, 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 incorporated into a pre-existing carbon-containing pool, cellular carbon-containing pools were prelabeled by growing the microorganisms in glucose-U-^{14}C and/or in a mixture of amino acids, each uniformly labeled with ^{14}C. Then ^{32}P uptake was monitored when cells were challenged with carrier glucose with or without glucose-U-^{14}C. After the period of phosphate uptake in response to challenge by exogenous glucose, cells were either extracted with NaOH or sequentially extracted with cold $HClO_4$ and NaOH.

   Cell extracts, after treatment with activated charcoal, were analyzed by column chromatography on Sephadex gels and on Dowex-1-formate. Fractions eluted from the columns were counted for ^{32}P and ^{14}C radioactivity. Techniques were employed which permitted elution of ^{32}P-containing fragments independent of ^{14}C-containing fragments. ^{32}P counts were resolved from ^{14}C counts by a computerized quench correction technique. The barium precipitable fraction of the charcoal treated extracts was determined.

   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 Findings:

1. About 30% 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 exogenous ^{14}C into the same cellular pools.

3. A substantial 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 polyphosphates--polymers of phosphoryl residues, free of carbon.

4. By Dowex-1-formate column chromatography a fraction containing ^{32}P and free of ^{14}C can be separated from the charcoal treated cell extracts. Its chromatographic behavior is consistent with its identification as polyphosphates. Rechromatography of this material upon thin layer plates, employing authentic polyphosphate standards, identifies a series of polyphosphates with chain lengths up to approximately 20. This indicates that polyphosphates of up to approximately 1800 molecular weight units are synthesized by these cells. Molecular sieve chromatography upon Sephadex gels supports this conclusion.

5. Data reported previously by Tanzer (Annual Report 1967) dealing with the fate of glucose metabolized by these streptococci, support this conclusion.

6. Growth studies presented as a separate report (NIDR-5), reveal that growth of these microorganisms in the mouth is slow, so suggesting that the classical conditions required for polyphosphate formation indeed exist in the streptococcal plaque in vivo.

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 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 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.

1. The influence of sucrose upon the accumulation of phosphate requires investigation. Older literature suggests differences in phosphate accumulation by dextran-forming organisms in the presence of sucrose, as compared to accumulation in the presence of glucose.

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 difference, 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.

Publications:

1. Tanzer, J.M., Krichevsky, M.I. and Chassy, B.: Separation of polyphosphates by anion exchange thin layer chromatography. J. Chromatog. 38: 526-531, 1968.

2. Tanzer, J.M., Krichevsky, M.I. and Keyes, P.H.: The coupling of phosphate accumulation to acid production by non-growing streptococci. J. Gen Microbiol. in press.

3. Tanzer, J.M., Krichevsky, M.I. and Keyes, P.H.: The metabolic fate of glucose catabolized by a washed stationary phase caries-conducive streptococcus. Caries Res. in press.

Serial No. NIDR-5 (68)
1. Office of the Director of Intramural Research
2. Environmental Mechanisms Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Growth Kinetics of Plaque-Forming Streptococci in the Presence of Sucrose

Previous Serial Number: None

Principal Investigator: Dr. J.M. Tanzer

Other Investigators: Mr. W.I. Wood, ADA Guest Worker and Dr. M.I. Krichevsky

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description:

Objectives:

The ultimate goal of this project is the definition of the *in vivo* growth characteristics of plaque-forming streptococci implicated in multisurface caries.

The immediate goal of this project is the characterization of the growth and regulation of growth of plaque and plaque-forming cells *in vitro*.

Methods Employed:

Two types of experiments have been carried out. 1) Intact plaques of caries-conducive streptococci were grown on sterile tared glass rods in the presence of complex bacterial growth medium containing 5% sucrose. The growth of the plaque, as indexed by its dry weight and DNA content, was monitored daily. 2) The growth of plaque-forming and non-plaque-forming streptococci was studied in complex bacterial growth medium to which various sugars had been added. Turbidity and DNA content of cultures were used as indices

of growth, and monitored minute to minute. Experiments were carried out in the presence of dextranase.

Major Findings:

1. Growth of intact plaques of dextran-forming streptococci is linear both on a dry weight and DNA basis and proceeds at about 6.8 μg dry weight/mm^2/day. It requires about 1.9 day for the dry mass of plaque to double. Thus plaque growth is slow.

2. There is no statistically significant evidence that the proportion of cells to extracellular products changes over 7 days of plaque growth. However, the data suggest that plaque may become less cellular as it grows for periods greater than 7 days.

3. Sucrose restricts plaque-forming streptococci from growing exponentially. Growth kinetics in the presence of sucrose are linear.

4. Plaque-forming streptococci in the presence of sugars other than sucrose and non plaque-forming streptococci in the presence of all sugars tested grow with classical exponential kinetics.

5. Linear growth of plaque-forming streptococci in the presence of sucrose is due to the synthesis of dextrans by these cells. Two lines of evidence support this conclusion: 1) non plaque-forming strains, which do not form dextrans in the presence of sucrose, grow exponentially in the presence of sucrose 2) plaque-forming strains, which form dextrans in the presence of sucrose and which grow linearly in the presence of sucrose, grow exponentially after the addition of dextranase.

Significance to Dental Research:

In the complex bacterial growth medium employed in these studies the conditions for streptococcal proliferation are probably much better than those existing in the mouth at the surface of the tooth. Therefore, in vivo streptococcal plaque growth should be even slower than the in vitro growth described here. Furthermore, this work has demonstrated that for the plaque-forming streptococci implicated in multisurface caries, the presence of sucrose, the requirement for plaque formation, specifically restricts the growth of these cells. These conclusions are supported by the common clinical observation that caries-associated plaque does not increase in mass many fold per day. It is simple to calculate that if an individual begins a day with 1 mg of adhesive streptococcal plaque on his teeth and if that plaque doubles its weight hourly,

assuming no oral clearance of these adhesive microorganisms, after 24 hours the teeth would be covered by almost 17 kilograms of plaque. Clearly this doesn't happen.

This work has established the reason for in vitro slow plaque growth--growth restriction of cells in the presence of sucrose due to dextran formation. Even if the oral fluids were the most ideal culture medium for plaque-forming streptococci, these cells could not grow exponentially in the presence of sucrose.

It can be hypothesized that the growth restriction results from a diffusion barrier imposed by the extracellular synthesis of dextran. However, linear growth cannot be explained by postulating a diffusion barrier surrounding individual cocci, since binary fission of such cells should produce exponential growth kinetics, although with slower generation time. Because growth in the presence of sucrose is linear, the data suggest that the diffusion barrier exists around clumps of cells, i.e., microcolonies. Furthermore, it is likely that within microcolonies there should be varying growth rates due to differential substrate availability and product accumulation.

Another portion of this report (NIDR-4), dealing with polyphosphate formation by these streptococci, indicated that polyphosphates are generally thought to be formed by poorly growing or nongrowing cells. The formation of polyphosphates by such sucrose-restricted slow growing streptococci is therefore suggested, and is projected for additional study.

The demonstration that plaque-forming streptococci can only grow slowly in the presence of sucrose serves to focus our attention in the study of the physiology of these cells in respect to caries. It suggests that study of the physiology of slow growing or non-growing cells is more relevant than study of exponentially growing cultures.

Proposed Course of Study

Growth of dextran-forming streptococci will be investigated with the goal of determining a) the basis for linear rather than exponential growth kinetics b) the actual diffusion restriction imposed by plaques upon the supply of critical metabolic substrates and endproducts in respect to caries.

Part B not included

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

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Sucrose Metabolism by Cariogenic Streptococci

Previous Serial Number: Same

Principal Investigator: Dr. B.M. Chassy

Other Investigators: Dr. J.M. Tanzer and Dr. S. Robrish

Cooperating Units: None

Man Years:
Total: 3/4
Professional: 3/4
Other: 0

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 (via isotopically labeled sucroses above) in cariogenic streptococci under a variety of growth conditions (i.e. under varying conditions of pH, substrate concentration, gas phase, oxidation potential, temperature, time, etc.) and 3) to evaluate the effect of exogenously supplied dextranase on sucrose utilization.

Methods Employed:

Sucrose (specifically labeled) was synthesized by a combination of enzymatic and chemical methods. These specifically labeled sucrose have heretofore not been available at as high an isotopic 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.

Apparatus is being constructed which will allow measurement of growth (as turbidity) in cultures using an experimental fiber optic probe. Included also will be temperature control, pH control, and control of substrate level. Facility of continuous measurement of pH, turbidity, substrate, gas phase, oxidation potential will be incorporated. Ultimately this system will operate on-line with computer feedback control of variables.

Major Findings:

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

2. Preliminary experiments are encouraging for the use of controlled growth apparatus in investigating the cariogenic streptococci.

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. To study the effect of various factors on growth and development of cariogenic streptococci (i.e. pH, inhibitors, substrate, etc.).

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 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

Publications:

1. Tanzer, J.M., Krichevsky, M.I. and Chassy, B.: Separation of polyphosphates by anion exchange thin layer chromatography. J. Chromatog. 38: 526-531, 1968.

Serial No. NIDR-7 (68)
1. Office of the Director of Intramural Research
2. Environmental Mechanisms Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Mathematical Models for Biological Growth

Previous Serial Number: None

Principal Investigator: Dr. S.A. Robrish

Other Investigators: Dr. M.I. Krichevsky

Cooperating Units: Washington Engineering Services Company

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description:

Objectives:

To analyze the details of a model for biological growth formulated by Mr. Frank Koenig of the Washington Engineering Services Company and to compare this model to some published data on bacterial growth.

Methods Employed:

Mathematical modeling by Mr. Frank Koenig of the Washington Engineering Services Company, contract number PH43-68-1001 in the amount of $22,651.

Major Findings:

Many of the major papers on the subject of bacterial growth in batch and continuous culture have been reviewed. New relationships have been derived which expand on the original equations of Koenig and make it possible to test this model from published data on bacterial growth or data from direct experiment.

Significance to Dental Research:

Mathematical models for bacterial growth will give us some useful ideas for the analysis of data taken in the detailed study of the growth of the cariogenic bacteria.

Proposed Course of Study:

The expanded model will be evaluated using published data or data which we will acquire with the device for the continuous monitoring of bacterial growth described in a previous section.

Part B not included

Serial No. NIDR-8 (68)
1. Office of the Director of Intramural Research
2. Environmental Mechanisms Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: The Nutrition of Oral Cariogenic Streptococci

Previous Serial Number: None

Principal Investigator: Dr. S.A. Robrish

Other Investigators: None

Cooperating Units: None

Man Years:

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

Project Description:

Objectives:

1. To establish defined minimal media for reliable growth of these organisms in order to assess their intermediary carbon and nitrogen metabolism.

2. To study the details of the growth patterns of these organisms on a variety of substrates.

3. To develop new methods for the study of the nutrition of microorganisms.

Methods Employed:

Standard microbiological methods.

Major Findings:

Strain SL-1 was chosen as a typically fastidious cariogenic streptococcus. Cystine has been identified as the constituent

necessary for reliable growth on a simplified medium consisting of yeast extract tryptone, and glucose. In addition to this the organisms can now be grown on a completely defined medium which, so far, is reliable for the continued growth of the organism. In the course of attempts to grow the organism on a completely defined medium, a detailed analysis of the minimal amino acid requirements of the organism is under way.

The construction and test of various components of a device for the growth of organisms under constant conditions of temperature and pH has proceeded. With this device we should be able to measure the turbidity of a culture continuously under highly controlled conditions. A prototype of a new fiber optic device is now being tested which will allow us to determine the turbidity of the culture continuously without the necessity for the pumping of the culture medium outside the initial container or withdrawing samples in order to determine the parameter.

In collaboration with Dr. Andre Leroy, BEIB, DRS, feasibility studies are underway on the design of a device to automate the determination of the nutritional requirements of microorganisms. Devices of three levels of sophistication are being considered: 1) a simple device which would duplicate the actions of a technician and be run with a maximum amount of intervention on the part of the investigator; 2) a programmed machine run by a process control computer with a limited amount of intervention by the investigator; and 3) a machine in which the computer would make most of the decisions and there would be almost no intervention on the part of the investigator. We have assembled the necessary data for the evaluation of the physicochemical parameters of the chemical constituents to be handled by such devices. In addition, economic analysis of these chemicals indicates that the cost of the chemical constituents for the first type of device is not limiting.

Significance to Dental Research:

Since a causal relationship has been shown between the formation of smooth surface dental caries and sucrose consumption a detailed study of the intermediary metabolism of sucrose and the consequent formation of a dextran polymer is potentially applicable to the problems of the dental profession. The development of a simple defined medium for the growth of these organisms will aid the interpretation of both the studies in the intermediary metabolism of the organisms and also the growth studies. A detailed study of the growth properties of these organisms may give us some insight into the potential pathological properties in the oral cavity. A detailed analysis of the nutritional properties of these organisms will give us an insight into their intermediary metabolism without going through many of the laborious processes of purified enzyme studies.

2

Proposed Course of Study:

1. The completion of the design and the start of the construction of the automated device for the determination of the nutrition of microorganisms. Since this type of device is applicable to a wide variety of problems in microbial nutrition in addition to those found in the organisms of importance to the dental profession, we hope to interest others in the use of the instrument.

2. A determination of the details of the intermediary metabolism of sucrose by the cariogenic streptococci using the specifically labeled sucrose preparations synthesized by Dr. Chassy (NIDR-6).

3. A detailed analysis of the growth properties of the cariogenic streptococci in liquid culture using the growth monitoring device.

Part B not included

Serial No. NIDR-9 (66)
1. Office of the Director of Intramural Research
2. Environmental Mechanisms Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Laboratory Information System

Previous Serial Number: NIDR-8

Principal Investigator: Mr. J.J. Wilson

Other Investigators: Dr. M.I. Krichevsky

Cooperating Units: Division of Computer Research and Technology

Man Years:

Total:	1 1/4
Professional:	1 1/4
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
Additional time sharing services have been evaluated. Two more

services have been determined to offer features useful to NIDR and service has been initiated.

2. Caries Studies on Rats
Forms were designed to facilitate computer data processing of caries data obtained from rats.

3. On-Line Laboratory Computer
A Honeywell 516 computer system for laboratory automation was selected. Working with DCRT personnel, the contract with Honeywell was negotiated. Software and hardware was designed to provide on-line acquisition of data from laboratory instruments. Programs are now being written for the computer system peripherals, for the data acquisition and for the processing of laboratory data. The specifications for the computer site were determined and the layout of the system was made.

Proposed Course of Study:

1. On-Line Applications
Software to support additional laboratory instruments such as spectrophotometers will be designed and written. Closed loop experimentation systems will be designed and implemented for the areas of:

1) neurophysiological experiments for determining optimum responses during micro-electrode recordings.

2) multiparametric growth experiments with cariogenic streptococci.

Interface of the 516 computer system with the NIH central computer center will be implemented. Software will be designed and written to provide communication between the computer systems.

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

Publications:

1. Wilson, J.J., Syed, D., DeLeo, J., Holsinger, W., and Krichevsky, M.I.: On line data acquisition for a bio-medical research laboratory. Proceedings of the Fall CAP Meeting. December 1968, pp. 77-88.

Report of the Neural Mechanisms Section
National Institute of Dental Research
Summary Statement

The Neural Mechanisms Section was recently established to study neural mechanisms in the oral and facial regions, especially those related to responses of the organism to its external environment. Investigators in the Section presently are pursuing two major problems: 1) peripheral and central neural mechanisms of oro-facial sensation mediated by the trigeminal system, with particular emphasis on responses to painful stimuli, and 2) sensory interaction in integrative centers of the brain and their participation in pain mechanisms.

The research studies in the trigeminal system are concerned with sensory transmission in two subnuclei of the trigeminal brainstem nuclear complex: the main sensory nucleus and nucleus caudalis. Previous findings by others after punctate neurosurgical lesions in these subnuclei indicate that the main sensory nucleus relays tactile sensation and that fibers subserving pain and temperature are relayed primarily in nucleus caudalis. However, previous work in this and other laboratories has failed to reveal many cells in nucleus caudalis that can be classified as exclusively "nociceptive" in terms of response characteristics. The present studies were designed to further assess functional differences which might explain the different roles of these two subnuclei in the mediation of facial cutaneous sensation.

Using neurophysiological techniques and recording the electrical activity of single cells in the face area of the cat cerebral cortex, direct axon projections to the main sensory nucleus and nucleus caudalis have been identified. Many of those cortical cells projecting directly to the trigeminal brainstem nuclei also received input from the face and mouth and responded to stimulation of peripheral trigeminal nerve fibers (infraorbital nerve). In addition, when these cells were stimulated via their axon projections in the trigeminal brainstem nuclei, they exhibited an increase in excitability if axon stimulation was preceded by stimulation of peripheral trigeminal fibers. Cortical cells projecting to nucleus caudalis showed an increase in excitability which usually was shorter in duration than the effects in the main sensory nucleus. This change in excitability demonstrated that cortical fibers terminating in the main sensory nucleus and nucleus caudalis were subject to presynaptic depolarization by trigeminal nerve stimulation.

In summary, a rapidly conducting feedback loop has been identified wherein cortical cells, many of which have an oro-facial input, project directly to trigeminal brainstem nuclei. Previous work has indicated that activity in these cortical cells results in presynaptic depolarization of trigeminal afferent fibers, and the present results demonstrate the reciprocal mechanism, where the axons of these cortical cells are themselves subject to presynaptic modification by activity in trigeminal afferent fibers.

These data further elucidate mechanisms by which cutaneous input may be modified 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, 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 above studies offer physiological data that may help explain these phenomena.

In collaboration with the Laboratory of Histology and Pathology, the fine structural features of trigeminal brainstem nuclei are being investigated. These correlated cytological and physiological studies have been most fruitful, and offer considerable future promise of further elucidation of basic mechanisms of oro-facial sensation.

The physical and affective components of painful sensation involve 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 association areas of the cat cerebral cortex are responsive to diffuse visual, auditory, and somatic stimuli. Recent studies in the Neural Mechanisms Section have demonstrated that at least one of these areas, the anterior part of middle suprasylvian gyrus, is equipped to detect specific details about visual stimuli. There appear to be three distinct groups of visually responsive cells in this cortical area, one responding to changes in illumination, a second responding to rapid movement of objects in any direction, and a third responding preferentially to oriented edges moved in specified directions. Cells in the first group become unresponsive when visual cortex is transiently depressed, whereas the latter two groups of cells continue to respond during depression of visual cortex. The results suggest that this association area plays a role in visual attention and orientation.

Presumably auditory and somatic input to association cortex will prove to be organized with comparable specificity. Such specific details about different modalities of input may provide these association areas with the sensory information necessary for their participation in the organism's awareness of painful stimuli and pain experience.

Serial No. NIDR-10 (66)
1. Office of the Director of Intramural Research
2. Neural Mechanisms Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

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

Previous Serial Number: NIDR-1

Principal Investigator: Dr. R. Dubner

Other Investigators: Dr. Barry J. Sessle and Dr. S. Gobel, Laboratory of Histology and Pathology, NIDR

Cooperating Units: None

Man Years:

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

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 influence of central and peripheral inputs on these cells.

Methods Employed:

Single neuron activity was examined with extracellular microelectrode recording techniques in the sensorimotor cortex of cats anesthetized with chloralose. An axon projection from the cortical cells to the trigeminal brainstem nuclear region was

identified by antidromic excitation of the cells with stimuli applied to bipolar electrodes located in the trigeminal main sensory nucleus and nucleus caudalis. An oro-facial input to the cortical cells was determined by stimulation of the infraorbital nerve and by locating the peripheral receptive field of cells with a fine brush or a blunt probe. Presynaptic depolarization of the corticofugal axons by trigeminal afferent stimulation was determined by noting any change in the cell's excitability during the presence of a conditioning infraorbital nerve (IO) stimulus.

Major Findings:

A direct axon projection to the main sensory nucleus, nucleus caudalis, or to both was identified in 75 cortical cells. The majority of these were located in an area just rostral to the peak face projection region of somatosensory cortex. An IO input was identified in 32 of the antidromically activated cells. Most of the cells antidromically activated from the main sensory nucleus and tested with IO conditioning stimulation, showed an increase in excitability above control levels, indicating presynaptic depolarization of corticofugal terminals by IO afferents. The effect was usually maximal at a conditioning-test interval of 40 msec and often had a duration lasting 300 msec or more. Cells projecting to nucleus caudalis exhibited an increase in excitability which usually was shorter in duration (100 msec or less) than the effects in the main sensory nucleus.

Significance to Dental Research:

We have presented evidence that corticofugal fibers to the trigeminal brainstem nuclei, in addition to producing presynaptic depolarization of trigeminal primary afferent fibers, are themselves subject to presynaptic depolarization by trigeminal nerve stimulation. Moreover, a rapidly conducting feedback loop has been identified wherein cortical cells receiving short latency cutaneous input have axons projecting directly to the trigeminal brainstem nuclei.

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 1) primary afferent depolarization of single trigeminal fibers; 2) interactions between nucleus caudalis and the main sensory nucleus; and 3) patterns of activity initiated by large and small fiber components.

Part B

Publications:

1. Dubner, R., Sessle, B.J. and Gobel, S.: Presynaptic depolarization of corticofugal fibres participating in a feedback loop between trigeminal brainstem nuclei and sensorimotor cortex. Nature. in press.

2. Dubner, R.: Peripheral and central input to the main sensory trigeminal nucleus of the cat. In Bosma, J.F. (Ed.): Oral Sensation and Perception II. Illinois, C.C. Thomas. in press.

Serial No. NIDR-11 (64)
1. Office of the Director
   of Intramural Research
2. Neural Mechanisms Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

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

Previous Serial Number: NIDR-2

Principal Investigator: Dr. B. Dow

Other Investigators: Dr. R. Dubner

Cooperating Units: None

Man Years:

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

Project Description:

Objectives:

The association areas of the cat cerebral cortex are responsive to visual, auditory, and somatosensory stimuli. They are thought to play a role in higher cognitive functions of the brain, though the nature of this role has remained elusive. This study deals with one such association area in the cat, the anterior portion of the middle suprasylvian gyrus (AMSS), which our previous work has shown to be more responsive to visual than to auditory or somatosensory stimulation. In addition, we have recently demonstrated restricted visual fields and binocular interaction in cells of this cortical area. There is thus clear evidence of organized visual input to an association area in the cat cerebral cortex. In the present work a variety of moving and stationary visual stimuli are used to study receptive field properties of AMSS cells, and also to determine the dependence of these response properties on primary visual cortex.

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^{o}-8^{o} in diameter) and variously oriented rectangular bars ($1/4^{o}$-2^{o} width, 2^{o}-6^{o} length), as well as straight edges of variable length (up to 20^{o}). As a means of defining the dependence of cell response properties on input from the primary visual system, selected cells were studied before, during, and after the transient depression of visual cortex by topically applied potassium chloride.

Major Findings:

Of a total of 212 cells, approximately two-thirds responded to visual stimulation. Extensive receptive field mapping done on 92 cells permitted the identification of three subgroups: 1) Edge detector cells give brief spike bursts to specifically oriented edges, moved in preferred directions at speeds of 50^{o}-150^{o}/sec across their 5^{o}-15^{o} diameter receptive fields. 2) Movement detector cells respond to movement of any stimulus in any direction across their 20^{o}-50^{o} diameter receptive fields, providing the movement is fast enough (50^{o}-150^{o}/sec). They also give brief on/off responses to small stationary spots flashed on to their receptive fields. 3) Illumination detector cells fail to respond with any reliability to moving stimuli, but give on/off responses to stationary spots over 10^{o}-30^{o} diameter receptive fields. Increasing the size and/or intensity of the stimulus enhances the response, indicating spatial summation without any detectable surround inhibition. Following application of KCl to visual cortex, 11 of 13 illumination detector cells showed diminished responses to visual stimuli, whereas all 13 movement and edge detector cells tested continued to respond. The results suggest that there are two populations of visual cells in this area, one responding to moving stimuli and essentially independent of primary visual cortex, the other responding to changes in illumination and dependent on primary visual cortex.

Significance to Dental Research:

The physical and affective components of painful sensation involve 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.

Our studies have demonstrated that at least one association area of cat cerebral cortex is equipped to detect specific details about visual stimuli, thus weakening the hypothesis, advanced by several workers, that all association areas of cat cerebral cortex receive identical inputs and are devoid of sensory specificity. Presumably, auditory and somatosensory input to association cortex will prove to be organized with comparable specificity. Polysensory convergence of organized input from multiple peripheral and central sources provides the association cortex with integrative capabilities necessary for participation in the perception of sensations such as pain, taste, and olfaction, where affective phenomena play a prominent role.

Proposed Course of Study:

Attention is now being directed to the pathways mediating visual input to this region of association cortex. Acute and chronic ablation studies involving both adult cats and visually inexperienced kittens are currently being initiated. Continuing physiological studies will be combined with recently developed anatomical methods to determine the nature of the interaction of this cortical region with other visual centers, most notably primary visual cortex and superior colliculus.

Techniques are being developed for studying single cell responses with chronically implanted electrodes in awake cats. Computer methods will be employed to analyze time distributed cellular events 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. Dow, B.M. and Dubner, R.: Visual receptive fields and responses to movement in an association area of cat cerebral cortex. J. Neurophysiol. in press.

2. 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.

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

With the creation of the Microbial Physiology Section, all personnel and projects of the Laboratory of Microbiology were consolidated for the first time under respective section affiliations. This organizational arrangement has produced important advantages in clearer definitions of mission, esprit de corps, and collaborative projects. Consequently, this summary is presented according to the Section designations.

Gnotobiotics Section

The Goal of the Gnotobiotics Section has been to identify and analyze the manifold parameters whose ecological interplay in the host-microbiota-environment complex in the oral cavity produces oral disease, primarily dental caries but also periodontal disease and pulpal pathosis. Accordingly, the Section has used not only germfree and gnotobiotic animals but also has investigated experimental infections in conventional animals, related problems in laboratory microbiology, and epidemiologic bacteriology in humans. Research initiated collaboratively a decade ago by this Section and the Laboratory of Histology and Pathology has built up the first impressive body of evidence identifying an oral streptococcus, denominated Streptococcus mutans, as one specific etiologic agent of dental caries. This bacterium satisfies Koch's classic criteria of pathogenetic significance: it occurs regularly in carious lesions of humans, hamsters, and rats; it can be isolated directly in pure culture from such lesions; these pure cultures induce typical caries when inoculated into the oral cavity of susceptible hosts, i.e., hamsters and both conventional and gnotobiotic rats; and the cycle can be reproduced from the experimental infections so induced. Related studies have identified an oral filamentous organism, denominated Odontomyces viscosus, as the specific microbial component of subgingival plaque accumulation, periodontal pathosis, and root-surface caries in hamsters; its implications for other animals and for humans are under continuing investigation.

In comparison with other carbohydrates, sucrose has been generally recognized to be an unusually and specifically cariogenic dietary constituent. Coincidentally, the one characteristic found so far that distinguishes S. mutans significantly from a wide variety of other oral acidogens is its property of converting sucrose to an extracellular polysaccharide (dextran) of high molecular weight, while freeing the fructose moiety of sucrose for its nutrition. The dextran has several properties that conduce to accumulation of cariogenic plaque on the teeth: it adsorbs readily to dental enamel, it forms insoluble complexes with various proteins and, perhaps most important of all, it causes S. mutans to agglutinate. This reaction is quite specific for S. mutans; none of a variety of noncariogenic oral streptococci and other bacteria tested, even some that produce dextrans, is so agglutinated. As a useful by-product, this reaction provides an extraordinarily sensitive test for identifying S. mutans.

Streptococcus mutans is typically associated with caries of the smooth surfaces of teeth, although it can induce lesions on occlusal surfaces as well. Thus, the NIH Black Rat normally does not develop caries lesions on the smooth surfaces even when maintained on a high-sucrose diet conducive to this type of lesion in other strains of rats. This resistance might indicate a genetic trait of the teeth, or it might indicate that the Black Rat normally does not harbor organisms which can colonize and attack the smooth surfaces of the dentition. To test these possibilities, a colony of germfree Black Rats was established, then monoinfected with S. mutans and maintained on a high-sucrose diet. These animals developed a high incidence of caries lesions on the smooth surfaces and in the dental sulci as well.

Even when inoculated with S. mutans, however, the Black Rat develops only about half as much caries as the more commonly used Osborne-Mendel rat. This difference has been shown to be attributable to a different habit of alimentation. The Black Rat eats and drinks about as much as the Osborne-Mendel rat but spends only half as much time doing so; that is, the cariogenic diet is in the oral cavity for a shorter time. (It is well established, in humans as well as in rodents, that reducing the frequency of eating reduces the incidence of caries.) When all test animals were placed on a regimen forcing them to eat a fixed large number of small meals a day, the difference between the two strains disappeared; in fact the Black Rats now developed rather more smooth surface caries than did the Osborne-Mendel rats.

This is one of only a handful of cases where the mechanisms of "genetic resistance " and "genetic susceptibility" to caries can be explained; here they are attributable to two traits, relative capacity to harbor S. mutans under usual conditions of laboratory maintenance and relative frequency of alimentation.

Like S. mutans, the specific cariogenicity of sucrose is associated mostly with smooth surface caries. This distinction is emphasized by recent studies in which high-sucrose diets were fed to rats only intermittently, sucrose being replaced ad interim either by equivalent mixtures of glucose and fructose or by a hydrogenated starch preparation. In all cases, the substitutions resulted in marked reductions of smooth surface caries but only an occasional animal showed a decrease of sulcal lesions. Nevertheless, these results encourage the hope that analogous substitutions might effect a significant reduction in human caries.

Though S. mutans has been isolated from the mouths of a majority of older children and adults in widely separated loci in Sweden, Colombia, and the United States, we lack information as to its contagiousness, that is, the frequency with which it is transmitted from human carriers and established in the mouths of noncarriers, particularly young children. Accordingly, with the cooperation of a population of 600 Coast Guard families living on Governors Island, New York, a study has been initiated which should answer this important question. Several years may be needed to complete this survey but the results to date justify confidence that the experimental plan will provide the desired information

Immunology Section

Current investigations in the Immunology Section are aimed at (1) the role of serum complement in the inflammatory response induced with endotoxin and antigen-antibody complexes and (2) factors regulating immunological tolerance and the

immune response. This work resulted in the publication of several manuscripts and two review articles concerned with the role of complement in the mechanisms of action of bacterial endotoxins.

With a steady growth of knowledge on the chemistry and reaction mechanisms involved in the sequential activation of the nine known complement components, it has been possible to obtain in relatively pure form biologically-active cleavage products from the third and fifth components of guinea pig and human complement. Such cleavage products can be derived from complement by activation with endotoxic lipopolysaccharide or antigen-antibody complexes. It has been shown in our laboratory in collaboration with scientists at the Johns Hopkins Medical School that a small molecular weight (15,000) peptide, derived as a split product from C5 after its activation by endotoxin or an antigen-antibody complex, has multiple effects on host tissue, i.e., chemotactic for polymorphonuclear leukocytes, smooth muscle contracting capabilities (anaphylatoxic) and permeability increasing properties. It is noteworthy that all of these properties may reside on the same molecule. A single factor which influences a number of biologic processes might be expected to act upon some cellular function common to all the cell types affected by it. It should therefore be possible to determine the biochemical events mediating such activities as chemotaxis and smooth muscle contraction. Understanding the biochemistry of this process might provide further insight into the general mechanisms involved in the inflammatory response. This in turn will be vital to a clearer understanding of mechanisms involved in such immunopathological phenomena as endotoxin action, graft rejection, and allergic inflammation characteristic in gingival diseases.

With the increasing interest in the feasibility of tooth and other oral tissue transplantation across weak and strong histocompatibility barriers, much effort has gone into studies on immunosuppressive agents. Antilymphocyte serum (ALS) is a potent immunosuppressive agent. One of the immediate problems with this biological reagent is that the host readily forms antibodies to the γG fraction of ALS and limits its usefulness because of anaphylatic-type reactions which develop after prolonged administration in the human. We have reported from our laboratory that a state of immunological tolerance to rabbit gamma globulin prolongs the effectiveness of rabbit anti-mouse lymphocyte serum in adult mice. That is, antibodies do not readily develop to ALS and skin allografts are prolonged in mice rendered tolerant to rabbit γ-globulin. In collaboration with investigators at the University of Minnesota Medical School, similar techniques are being employed in patients being treated with horse anti-human lymphocyte serum; patients are being administered a tolerogenic injection of normal horse γG prior to the administration of ALS. The investigators at Minnesota are encouraged to proceed with the studies because we have recently shown that tolerance to a protein antigen (human gamma globulin) can be established by similar techniques in immunologically competent primates (monkeys). Thus, these techniques developed in our laboratory could be used in a number of clinical situations where ALS is employed as an immunosuppresaive agent in the grafted patient.

The lactic dehydrogenase virus (LDV) was the first virus demonstrated to behave as an immunological adjuvant in the mouse. Continuing studies over the past year have shown that Venezuelan equine encephalitis virus (VEE) has adjuvant

characteristics in mice and guinea pigs. Infection of mice with VEE prevents the induction of tolerance to human gamma globulin and converts a tolerance-inducing stimulus into an immunity-inducing stimulus. However, unlike LDV, which has been shown to inhibit skin allograft rejection and the graft versus host reaction, VEE has no effect on the survival skin allografts. Thus, these virus infections have different effects on cell-mediated immune reactions. The ability of LDV and VEE to act as adjuvants may thus lead to useful models in studying the possible role of virus infections in autoimmune phenomena.

Microbial Physiology Section

Studies by members of the Microbial Physiology Section have dealt with an assessment of certain fermentation pathways and their regulation in various lactic acid bacteria. In addition, the taxonomic relatedness among species of the genus Lactobacillus have been studied by comparing a variety of phenotypic characteristics.

Group D streptococci. Studies on the fermentation of glucose, gluconate, and malate by S. faecalis have led to a fairly comprehensive understanding of both the degradative pathways involved and the factors that operate to regulate them. This organism has been found to have a constitutive complement of enzymes for degrading glucose by both the Embden-Meyerhof and hexosemonophosphate pathways. The former pathway serves primarily to supply energy (ATP), whereas the latter functions primarily to generate NADPH for reductive biosynthetic reactions and to supply pentose for DNA and RNA synthesis.

The flow of carbon through one or the other of these pathways has been found to be modulated by the intracellular concentration of a single small molecule, fructose-1, 6-diphosphate (FDP). At one concentration, this glycolytic intermediate both activates the lactate dehydrogenase (LDH) and inhibits the 6-phosphogluconate dehydrogenase (6PGD). Under the conditions, glucose is dissimilated primarily by the Embden-Meyerhof pathway. At a lower level of FDP, the LDH (which has an absolute requirement for FDP for activity) is inactive and the inhibition of 6PGD is relieved. Thus, glucose carbon can pass through the hexosemonophosphate pathway.

The mechanism by which FDP activates the LDH has been studied in some detail and our results indicate that the activation involves an FDP-mediated transition in state of the enzyme from a constrained to a more or less open configuration. The consequence of this transition is a greater accessibility of substrate and coenzyme binding sites to the reactants. Interaction of the enzyme with FDP appears to result only in a change in the tertiary structure of the protein and not a change in its quaternary structure. The molecular weight of the LDH is about 68,000 both in the presence and the absence of FDP.

Fructose-1, 6-diphosphate also plays a role in the regulation of malate metabolism. The aerobic dissimilation of malate by S. faecalis has been shown to be dependent upon the adaptive formation of a malic enzyme which catalyzes the initial cleavage of the substrate into CO_2 and pyruvate. This enzyme has been found to be inhibited by FDP (and certain other glycolytic intermediates), which probably explains why malate utilization ceases abruptly when glucose is added to the culture medium. Additional control of malate degradation is

exerted at the level of repression of malic enzyme synthesis by glucose or a product derived therefrom. A careful study of this phenomenon has revealed that it is actually an indirect consequence of the repression of a malate permeate. Without the permeate, the cell is unable to concentrate sufficient malate for induction of the malic enzyme. This appears to represent a novel and hitherto unreported mechanism for regulating cellular metabolism.

Streptococcus mutans. Specific biochemical steps involved in the fermentation of various compounds have been examined in a number of strains of cariogenic streptococci. These strains have included organisms of hamster, rat and human origin, although most of the work has been done with a human strain (S. mutans ATCC 10449).

Two hitherto unreported phenotypic characteristics have been found for this group of organisms: (1) They are unable to decompose gluconate. In vitro studies have indicated that this deficiency is due to the fact that these organisms lack the first two enzymes required for the dissimilation of gluconate by the hexosemonophosphate pathway. (2) These organisms are also incapable of growth on glycerol or pyruvate. S. mutans 10449 has been shown to lack fructose diphosphatase, which is a key enzyme involved in the synthesis of 6-carbon compounds from 3-carbon precursors. Its absence may well explain the inability of this organism to grow on pyruvate or glycerol.

Lactate dehydrogenases from eight different strains of S. mutans have been studied. The enzymes exhibit kinetic and physical properties that appear to be unique for this group of organisms. If further studies confirm this, these properties could be useful taxonomic aids and of possible diagnostic value for distinguishing cariogenic from noncariogenic streptococci. Several other biochemical characteristics have been examined in these organisms in an effort to resolve as many phenotypic traits as possible for future use in defining their taxonomic position among the streptococci.

Lactobacilli. A coordinated study of the physiology, intermediary metabolism and taxonomy of homofermentative and heterofermentative lactobacilli has been initiated. Results from preliminary studies have indicated distinct patterns of relatedness among certain groups of these organisms. These groupings are being compared with divisions based on the guanosine to cytosine ratios in the DNA of the organisms.

Virology Section

Over the past year we have continued to study in depth the mechanism of neutralization of sensitized herpes simplex virus (HSV) by complement, anti-γ-globulin, and antiviral antibody. Of major importance was the discovery (made in collaboration with Drs. Borsos and Rapp of NCI) that in the presence of the first component of complement, HSV, which had been sensitized with IgM anti-HSV, could be neutralized by the purified fourth component of complement and that neutralization could be enhanced by the addition of the purified second and third components of complement. These findings represent a hitherto unreported biological function for these components of complement, and support the hypothesis that sensitized virus is neutralized by the piling up of complement components on the surface of the virion. The use of sensitized virus and

of purified complement components is providing new insight into the mechanism of virus neutralization.

The high specificity of the interaction between anti-γ-globulin and the γ-globulin on the sensitized virion has allowed us to study antiviral immunoglobulins in greater detail. By the anti-γ-globulin technique we have been able to detect univalent fragments of antiviral antibody and to show allotypic differences between immunoglobulin molecules. Neutralization of sensitized virus by anti-γ-globulin has also led to the development of a new technique for measuring antibody to immunoglobulins which rivals and in certain cases exceeds the sensitivity of the red cell agglutination technique. This later technique has been regarded as one of the most sensitive methods for measuring antibody.

Previously we suggested that sensitization might in part be responsible for the chronic nature of certain virus infections. Studies completed this year on the kinetics of neutralization of sensitized HSV showed that univalent antibody fragments neutralized highly sensitized virus at a faster rate than did undigested divalent antiviral antibody. These findings suggest that univalent antibody fragments might prove useful in the neutralization of virus which is highly sensitized and relatively resistant to neutralization by undigested antiviral antibody.

Pertinent to the mechanism of virus neutralization was the demonstration this past year that sensitization significantly inhibited the rate at which HSV penetrated mammalian cells. In connection with the biological significance of sensitized virus it should be mentioned that Dr. Frank Dixon at the Scripps Foundation carried out experiments which suggest that in vivo sensitized virus (lymphocytic choriomeningitis virus) might be responsible for certain chronic diseases (i.e., glomerulonephritis).

A virus of the oral cavity which has been studied extensively by Mr. Warren Ashe is the rat submaxillary gland virus. Experiments were completed and published this last year on some of the physical and chemical properties of the hemagglutinin and antihemagglutinin of this virus and their incidence in gnotobiotic and conventional rats.

In collaboration with the Immunology Section, studies were continued on the effect of virus infections on the functional capacity of the immune system. We found that infection with lactic dehydrogenase virus (LDV) significantly inhibited the rejection of skin allografts and depressed the graft-versus host reaction. These findings together with our earlier studies on the effect of LDV on humoral antibody and immunological tolerance show that virus infections can profoundly alter the functional capacity of the immune system. In addition we showed for the first time that immunologically mature primates (monkeys) could be made tolerant to a soluble antigen (human γ-globulin). These findings suggest that with soluble transplantation antigens it might be possible to make mature primates tolerant to allografts of all types, including teeth.

Over the last 12 months a number of new techniques have been set up in our laboratory. For studies on the physical and chemical properties of sensitized virus we have labeled HSV with H^3 tymidine and purified the virus by sucrose gradient sedimentation. In addition, we are attempting to label antiviral antibody with I^{125}, and in collaboration with Dr. Howard Bladen we are studying by electron microscopy the interaction of ferritin labeled anti-γ-globulin with sensitized virus. For other physical-chemical studies on sensitized virus, polyoma virus offers certain advantages and the assay for this virus has been set up in our laboratory. Lastly, in an effort to rescue "latent" virus from patients with recurrent HSV infections we have set up a technique which can be used to fuse cells suspected of harboring "latent" virus with cells susceptible to infection with HSV. The fusion technique involves the use of inactivated Sendai virus as the fusing agent. This technique is also being used to study the transfer of information between antibody producing cells.

Serial No. NIDR - 12 (59)
1. Microbiology
2. Gnotobiotic
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Gnotobiotic Studies of Problems Relating to Oral Disease

Previous Serial Number: NIDR-9

Principal Investigator: Dr. R. J. Fitzgerald

Other Investigators: Dr. R. H. Larson

Cooperating Units: Forsyth Dental Center

Man Years:

Total:	5 1/4
Professional:	1
Other:	4 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 disease. Germfree, conventional and limited flora animals are used to investigate the role of these organisms in disease processes.

Major Findings:

1. Agglutination of cariogenic streptococci by dextran of high molecular weight (with Dr. R. J. Gibbons, guest scientist from the Forsyth Dental Center).

Addition of dextrans of molecular weights above 10^6 has been found to cause agglutination of all strains of cariogenic streptococci tested. Non-cariogenic organisms, even those which produce dextrans or levans, do not exhibit this type of reaction. Low molecular

weight dextrans, e.g., 10^4 m. w., do not induce agglutination. The amounts of high molecular weight (HMW) dextran required to agglutinate the cariogenic streptococci are extremely small; detectable agglutination has been obtained in cell suspensions containing an estimated 3 molecules of HMW dextran per cell.

2. Induction of smooth surface caries in Black Rats (with D. B. Fitzgerald and R. H. Larson). The NIH Black Rat normally does not develop smooth surface caries lesions even when maintained on Diet 2000, which is highly conducive to this type of caries in other strains of rats. Dr. Larson found earlier that superinfection of the Black Rat by oral inoculation of fecal microflora suspensions from caries active Osborne-Mendel rats resulted in the development of caries activity on the smooth surface areas as well as in the pits and fissures of the Black Rat's molar teeth. This suggested that the Black Rat normally lacked an organism or organisms which could colonize and attack the smooth surfaces of the dentition. We obtained the Black Rat in the germfree state by Cesarean section and foster suckling the young animals on germfree Sprague-Dawley rats. Progeny of this colony were monoinfected with a single cariogenic strain of Streptococcus mutans (K1-R) of human origin and maintained for 56 days on Diet 2000. This resulted in a high incidence of smooth surface caries activity in addition to lesions in the pit and fissure areas.

Significance to Dental Research:

The observations on the dextran induced specific agglutination of cariogenic streptococci suggest that these organisms have specific receptor sites, possibly the enzyme dextransucrase, which bind dextrans. If the dextran molecule is sufficiently large, two or more cells may bind to the same molecule causing agglutination. Two important implications may be derived: (1) that this phenomenon may be made the basis of a simple screening test for identifying potentially cariogenic organisms; and (2) it suggests a means by which cariogenic organisms may be selectively attracted to the tooth surface at the site of existing dextran-producing organisms and so build up plaque deposits.

The germfree Black Rat experiments show that a special type of microorganism must be present in the oral flora to induce smooth surface caries lesions whereas pit and fissure lesions may be produced both by this type of organism and other "normal" oral microorganisms. The implication is that the eventual control of dental caries may require approaches which can eliminate both types of microorganisms or which can selectively inhibit the cariogenic potential of each type.

Proposed Course of Project:

The principal investigator has retired from the U. S. Public Health Service. This project has been terminated as such, and selected phases have been incorporated in other projects.

Part B:

Publications:

1. Fitzgerald, R. J. Plaque microbiology and caries. Alabama J. Med. Sci. 5: 239-246, 1968.

2. Fitzgerald, R. J. Dental caries research in gnotobiotic animals. Caries Res. 2: 139-146, 1968.

3. Fitzgerald, R. J. Maturation of dental enamel in germfree and monoinfected Sprague-Dawley rats. Helv. Odont. Acta 12: 55-61, 1968.

4. Fitzgerald, R. J., and Jordan, H. V. Polysaccharide-producing bacteria and caries. In Harris, R. S. (Ed.): The Art and Science of Dental Caries Research. New York, N. Y., Academic Press, 1968, pp. 79-86.

5. Larson, R. H., and Fitzgerald, R. J. Caries development in the African white-tailed rat (Mystromys albicaudatus) infected with a streptococcus of human origin. J. Dental Res. 47: 746-747, 1968.

6. Kakehashi, S., Stanley, H. R., and Fitzgerald, R. The exposed germfree pulp: effects of topical corticosteriod medication and restoration. Oral Surg. Oral Med. and Oral Path. 27: 6067, 1969.

7. Gibbons, R. J., and Fitzgerald, R. J. Dextran induced agglutination of Streptococcus mutans and its potential role in the formation of dental plaque. J. Bacteriol. In press.

Serial No. NIDR - 13 (65)
1. Microbiology
2. Gnotobiotic
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

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

Previous Serial Number: NIDR-11

Principal Investigator: Dr. H. V. Jordan

Other Investigators: None

Cooperating Units: None

Man Years:

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

Project Description:

Objectives:

1. To study the occurrence and distribution of caries-inducing streptococci (Streptococcus mutans) in human populations and to establish patterns of their transmission.

2. To expand the scope of available model systems used to study bacteriological factors in periodontal disease and to determine the extent to which animal studies, particularly involving filamentous bacteria (Odontomyces viscosus), apply to the human situation.

Methods Employed:

Attempts are made to simulate oral diseases, or certain aspects of them, by the use of experimental animal models and in vitro laboratory models. Bacteria under study are labeled by making them resistant to antibiotics in order to follow their implantation and colonization in experimental animals and humans.

Part A (continued)

Major Findings:

1. Dental caries and specific streptococci. A project was initiated to determine whether active transmission of caries-inducing streptococci (S. mutans) occurs within family groups. Parents who harbor S. mutans as part of their normal oral flora are selected from the 600 Coast Guard families living on Governors Island, New York. Each volunteer family has at least one 2-3 year old caries-free child who has not yet acquired S. mutans. A strain of S. mutans is isolated from at least one parent subject, made resistant to streptomycin and reimplanted into the oral cavity of the donor parent. The subject's teeth are first polished with pumice paste in order to minimize competition of other plaque-forming bacteria. After inoculation the subjects eat candy for 2-3 days to facilitate implantation of the tagged organism. Antibiotic sensitivity patterns of the strains were determined to be the same before and after "tagging" except for the acquired streptomycin resistance.

   Strains of S. mutans were isolated from 11 mothers and one father out of a total of 25 families sampled. The 12 individuals were orally infected with their own"tagged"strain and 4 have retained the organisms for 30 days. All other subjects cleared the "tagged" organisms in less than 4 days. Weekly monitoring of the 4 families has not yet indicated any spread of the "tagged" organisms to uninoculated family members.

2. Relationship of Odontomyces viscosus and other filament-forming bacteria to periodontal disease. Studies on the etiologic relationship of O. viscosus and other filament-forming bacteria to periodontal disease has continued in an attempt to relate this disease model more closely to the human situation. Numerous strains of human origin have been tested in hamsters and gnotobiotic rats. To date no uniform pattern of plaque formation or disease has been noted. Strains were selected because of their similarity to O. viscosus or their ability to form artificial plaques in vitro as described in earlier reports. The human strains appear to implant with great difficulty and in reduced numbers in the rodents. Since implantation is greatly influenced by the diet of the host, a series of experiments were conducted to define dietary conditions which may control the implantation and colonization of this type of organism in rodents. A variety of carbohydrates could be substituted for sucrose in diet #2000. Implantation was actually delayed with sucrose in the diet when S. mutans was also present in the same animal. This may be due to the competition of S. mutans which is known to colonize in the presence of sucrose.

Part A(continued)

Starch, glucose or fructose-containing diets supported implantation of O. viscosus and development of periodontal disease in hamsters.

Collaborative experiments were conducted to aid Dr. Tempel's studies on the chemotactic effect of oral bacteria for leucocytes. Strains of O. viscosus and a levan-forming streptococcus (Gibbons) were used in these studies since these strains were implicated in periodontal disease in animals. Culture filtrates were found to be highly chemotactic. Complex bacteriological growth media contain unknown constituents with chemotactic activity. A defined medium with no chemotactic properties was developed for both types of organisms in order to avoid this difficulty.

Cooperative studies have continued 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. High titer antisera have been prepared against hamster and rat strains of O. viscosus. Antisera against strains from the hamster react in high titer with cells of O. viscosus from the rat and vice versa indicating their close serological relationship.

Teeth with root surface caries are preserved in transport medium (VMG II) and mailed to Bethesda. Bacteriological samples are obtained from the softened dentin in the depths of the lesion. Isolates are identified and filament-forming strains are tested with O. viscosus antisera. A number of strains show varying degrees of reactivity - none equaling the titer of the homologous reaction. Representative isolates of the reactive strains are currently being tested in gnotobiotic rats.

The high titer antisera have been coupled with fluorescein isothiocyanate and will be used to further identify filamentous bacteria isolated from cervical caries by the fluorescent-antibody technique. Sections of the teeth will be stained and examined by the FA technique in an attempt to identify specific bacteria in situ.

Part A (continued)

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. While it is still too early to speculate on possible patterns of spread of S. mutans in family groups, the study at Governors Island should put the concept of dental caries as a transmissible and infectious disease into some perspective. The study already indicates that S. mutans may be more widespread in human populations than was detected by previous surveys.

Studies on periodontal disease are expected to describe factors of importance which influence the ecology of the oral flora as it relates to this condition. Dietary studies on the implantation of O. viscosus in hamsters are pursued with this goal in mind. However, we have not yet been able to define factors which specifically enhance proliferation of these bacteria in competition with other plaque-forming organisms. The ultimate significance of these studies is to determine the true contribution of actinomyces-like organisms in human periodontal disease.

Proposed Course of Project:

Since the principal investigator will retire from the Public Health Service this year, the project is terminated as such. Much of the work will be continued by the principal investigator through grants and other mechanisms in a new research setting, and as part of other projects at NIDR.

Part B

Publications:

1. Fitzgerald, R. J., and Jordan, H. V.: Polysaccharide-producing bacteria and caries. In Harris, R. S. (Ed.): The Art and Science of Dental Caries Research. New York, N. Y., Academic Press 1968, pp. 79-86.

2. Jordan, H. V., Englander, H. R. and Lim, S.: Potentially cariogenic streptococci in selected population groups in the Western hemisphere. J.A.D.A. in press, 1969.

3. Jordan, H. V., Keyes, P. N., and Lim, S.: Plaque formation and the implantation of Odontomyces viscosus in hamsters fed different carbohydrates. J. Dent. Res. in press, 1969.

Part B (continued)

4. Jordan, H. V., Krasse, B., and Möller, A.: A method for sampling human dental plaque for caries-inducing streptococci. Arch oral Biol. 13: 919-927, 1968.

5. Krasse, B., Jordan, H. V., Edwardsson, S., Svensson, I., and Trell, L.: The occurrence of certain caries-inducing streptococci in human dental plaque material with special reference to frequency and activity of caries. Arch oral Biol. 13, 911-918, 1968.

Serial No. NIDR-14 (61)
1. Microbiology
2. Gnotobiotic
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

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

Previous Serial Number: NIDR-12

Principal Investigator: Dr. R. H. Larson

Other Investigators: Dr. R. J. Fitzgerald

Cooperating Units: University of Zürich, Zürich, Switzerland; Royal School of Dentistry, Stockholm, Sweden

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 animals.

Methods Employed:

Conventional methods for animal experimentation have been used. In addition use has been made of two types of special equipment (1) to record eating and drinking habits of rats and (2) automatic feeding equipment which forced rats to eat according to a programmed pattern. Detailed patterns of caries activity have been carefully determined in relation to the variations in the experimental procedure.

A method of recording scoring results has been devised which facilitates transfer of detailed information to punch cards for computer analysis.

Major Findings:

Studies of the interrelationship between host factors, diet and bacterial infection have been continued. It was shown that differences in eating habits was partly responsible for the widely different levels of caries development normally shown by the Osborne-Mendel rat and the NIH Black rat. In addition, the level of smooth surface caries was significantly higher in animals fed a sucrose diet continuously than in those fed the sucrose diet intermittently with the same diet except that sucrose was substituted by another sugar or a starch product.

(A) Influence of a strain specific eating pattern on the development of caries. In Experiment I, Osborne-Mendel and NIH Black rats were ad libitum fed the same diets, the average eating time per 24-hour periods was 96 minutes for the BRs and 213 for the O-Ms. The levels of caries development showed similar differences; BRs 0.3 lesions in the fissures and 0.0 on the smooth surfaces and the O-Ms 5.6 and 1.1, respectively. In Experiment II, the same high frequency eating pattern, of 36 meals per day, was imposed on the BRs and the O-Ms with the aid of an automatic programmed feeding machine. Under this feeding program the BRs showed a highly significant increase in caries over the ad libitum fed controls, with an increase in fissure lesions from 0.3 to 3.5 and smooth surface lesions from 0.0 to 3.4. The program fed O-Ms showed no increase in the fissures lesion over the controls 5.6 and 5.3 but showed an increase on the smooth surfaces from 1.1 to 2.8 lesions. Thus, when the two strains of animals were fed the same number of meals per day the BRs actually developed a higher level of smooth surface lesions (3.4) than the O-Ms (2.8).

(B) Influence of intermittent feeding of sucrose substitutes on the development of dental caries in the rat. When Osborne-Mendel rats were fed Diet 2000 and infected with plaque-forming caries-active organisms (K1-R) and were given intermittent feedings of diets in which various sugars and starches or fasting periods were used to replace feedings of the sucrose diet, there was a significant decrease in smooth surface caries development in comparison to the controls continuously fed Diet 2000, containing 56% sucrose. In Experiment I, sucrose substitutes were provided for half the time throughout the experiment, changing diets every two or three days. The number of carious areas on the smooth surfaces was 56.6 for the controls and varied for those on the sucrose substitute diets from 50.0 ($P<0.05$) for those on dextrose plus fructose to 3.8 ($P<0.001$) for those on Ca 6563, a hydrogenated potato starch. In Experiment II the substitute diets were fed only during the first week of the test period during the time that the caries-active organisms were being implanted. This procedure was even more effective in the reduction of caries in

that the controls developed 48.9 carious areas on the smooth surfaces; while those on substitute diets ranged from 32.6 ($P<0.001$) for those on Ca 6563 to 15.1 ($P<0.001$) for those on dextrose plus fructose. In Experiment III the substitute diets were given only after the week of implantation 4 nights per week, so as to provide approximately half the intake. The controls averaged 48.9 carious areas on the smooth surfaces, while those on substitute diets varies from 32.6 ($P<0.001$) for those on Ca 6563 to 15.1 ($P<0.001$) for those on dextrose plus fructose. Only an occasional group of animals in either experiment showed a decrease in sulcal lesion. Thus it appears that any interruption in sucrose intake is associated with a decrease in caries activity on the smooth surfaces, but is less effective in the reduction of sulcal lesions.

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. The results of the studies reported here serve to reinforce the results of previous findings that a reduction in sucrose, both in actual amounts consumed and in length of eating periods, offers great possibilities as a practical method for the reduction of human caries.

Proposed Course of Project:

There is considerable information to show that proper dietary habits may be associated with a decrease in caries development in both experimental animals and in man. It appears that much of the influence of these factors is mediated through influence on the oral flora. Yet very little really definitive work has been done in this area. Now that certain "highly cariogenic" organisms have been identified it will be possible to study the effect of specific dietary components on the establishment and subsequent activity of these organisms. Such an approach should offer leads in the search for dietary factors which may be of value in the control of human caries

Part B

Publications:

1. König, K. G., Larson, R. H., and Guggenheim, B. A.: A strain-specific eating pattern as a factor limiting the transmissibility of caries activity in rats. Arch. Oral Biol. 14: 91-104, 1969.

2. Larson, R. H.: Tooth age and caries susceptibility. In Harris, R. S. (Ed.): The Art and Science of Dental Caries Research. New York, N. Y., The Academic Press, 1968, pp 257-275.

3. Larson, R. H., and Fitzgerald, R. J.: Caries development in the white-tailed rat (Mystromys albicaudatus) infected with a rodent streptococcus. J. Dent. Res. 47: 746-749, 1968.

4. 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. Dent. Res. 47: 704-709, 1968.

Serial No. NIDR-15 (65)
1. Microbiology
2. Immunology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Immunological Mechanisms in Oral and Systemic Disease

1. Role of complement in Inflammation: Mechanism of Antigen-antibody and Endotoxin-Induced Inflammation.

2. Biological Effectors of Immune Response and Immunological Tolerance.

Previous Serial Number: NIDR-14

Principal Investigator: Dr. S. E. Mergenhagen

Other Investigators: Dr. R. Snyderman
Dr. R. J. Howard
Dr. W. A. Hook
Dr. T. R. Tempel (guest worker)

Cooperating Units: Dept. of Microbiology, Johns Hopkins University School of Medicine; Dept. of Pediatrics and Surgery, University of Minnesota School of Medicine; Pathology Division, U. S. Army Research Institute of Infectious Diseases, Ft. Detrick, Maryland; Department of Microbiology, University of Miami School of Medicine.

Man Years:

Total: 9 1/2
Professional: 5
Other: 4 1/2

Project Description:

Objectives:

1. To investigate immunological and other mechanisms by which oral and other microbial products incite tissue damage.

2. To investigate the role of cleavage products of complement components in inflammation.

3. To study the mechanism of action of viruses and other biological effectors on the immune response and on immunological tolerance.

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 a 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 complement with endotoxin and antigen-antibody complexes.

   (a) Cleavage products with chemotactic and anaphylatoxic properties.

   During the interaction of endotoxin or antigen-antibody complexes with the complement system various biologically active peptides are cleaved from the terminal components of complement. We have recently proposed that the complement system may be an important mediator of certain pathological effects which follow the introduction of endotoxin into a number of animal species.

   Upon activation of complement in guinea pig serum by endotoxin (or antigen-antibody complexes) a factor chemotactic for polymorphonuclear leukocytes is generated. This chemotactic factor has an approximate molecular weight (MW) of 15000. Purified radiolabelled guinea pig C'3 and C'5 were utilized to determine if the 15000 MW chemotactic factor generated in serum was derived from either of these two components. Gel filtration elution profiles of the reaction mixtures reveal that both C'3 and C'5 are cleaved in endotoxin-treated serum. Moreover there was an identity of the 15000 MW chemotactic factor with a cleavage product of C'5 but not C'3. In addition, rabbit anti-guinea pit C'5 but not anti-C'3 significantly inhibited the activity of the 15000 MW chemotactic factor.

It is furthermore of interest to note that when sensitized sheep erythrocytes (EA) were interacted with purified guinea pig complement components C'1, C'4, C'2, C'3, followed by C'5, a product with a MW of 15000 was cleaved from C'5. The product cleaved from C'5 had both smooth muscle contracting (anaphylotoxin) and chemotactic activities. These activities could not be disassociated by sucrose density gradient ultracentrifugation or polyacrylamide gel electrophoresis. It is noteworthy and should be stressed that the chemotactic and anaphylatoxic properties of the C'5 split product may reside on the same molecule. A single product with the ability to increase vascular permeability, contract smooth muscle, and induce neutrophil chemotaxis might be expected to be an important mediator of the inflammatory process. Indeed, ongoing experiments have shown that a semipurified preparation of the 15000 MW chemotactic factor caused increased permeability and neutrophil accumulation in rabbit skin in vitro. Further studies using purified complement component cleavage products will provide additional insight into the role these factors play in the inflammatory response.

(B) Complement profiles in human disease: correlations with in vitro models. In collaborative studies with the University of Minnesota, classical hemolytic complement component activities and several complement-dependent functions were measured in children with acute glomerulonephritis (AGN), systemic lupus erythematosus (SLE) and hypocomplementemic chronic glomerulonephritis (HCGN). Contrasting complement profiles were seen in SLE on one hand and in AGN and HCGN on the other. A complement profile similar to that in SLE appears in vitro when immune complexes are incubated with fresh guinea pig serum. By contrast incubation of guinea pig serum with lipopolysaccharide (endotoxin) resulted in a complement fixation profile more similar to that seen in AGN and HCGN. These models represent alternate pathways for activation of the terminal complement components and may well be applicable to the ways in which complement is consumed and in the mechanism of tissue destruction seen in patients with systemic lupus erythematosus and glomerulonephritis.

2. Neutrophil chemotactic factors elaborated by oral bacteria. A prominent feature of acute gingivitis is the accumulation of polymorphonuclear leukocytes in the gingival tissue with an eventual migration of such cells into the gingival sulcus area. It is now apparent that potent chemotactic factors can be elaborated during antigen-antibody or endotoxin-mediated inflammatory reactions via the complement system. In addition, certain strains of bacteria elaborate low molecular weight substances which have potent chemotactic activities. Two

strains of oral bacteria, Streptococcus sp. and Odontomyces sp., associated with a form of periodontal pathosis in the rodent, were examined for the production of factors chemotactic for neutrophils. Both organisms elaborate extracellularly a potent low molecular weight factor chemotactic for neutrophils in vitro. Factors which appear identical to those from bacteria by analysis with Sephadex G-10 chromotography can be found in saliva and the soluble portions of subgingival plaque. Preliminary characterization of the bacterial chemotactic factor indicates that it may be a nucleotide or similar type substance.

3. Biological effectors of immune response.

(A) Antilymphocyte Serum and immunological tolerance in primates. Antilymphocyte serum (ALS) is a potent immunosuppressive agent. One of the immediate problems with this biological reagent is that the host readily forms antibodies to the γG fraction of ALS and limits its usefulness because of anaphylactic-type reactions which develop after prolonged administration in humans. We have reported from our laboratory that a state of immunological tolerance to rabbit gamma globulin prolongs the effectiveness of rabbit anti-mouse lymphocyte serum in mice. That is, the ALS is not catabolized rapidly, antibodies do not develop to ALS, and skin allografts are prolonged in mice rendered tolerant to rabbit γ-globulin. In collaboration with scientists at the University of Minnesota, similar techniques are being employed in patients being treated with horse anti-human lymphocyte serum. Thus far the results are very encouraging; patients given a tolerogenic injection of normal horse gamma globulin prior to ALS administration show very little antibody to ALS, and skin grafts are prolonged as compared to the controls being treated with ALS but not given a tolerogenic stimulus. Scientists in Minnesota were further encouraged to proceed in humans because we have recently shown that tolerance to a protein antigen (human gamma globulin) could be established in immunologically competent primates (monkeys). These procedures could be used in a number of clinical situations where ALS is employed as a therapeutic agent in the grafted patients.

(B) Influence of virus infections on immunity. The lactic dehydrogenase virus (LDV) was the first virus demonstrated to behave as an immunological adjuvant in the mouse. Continuing studies over the past year have shown that Venezuelan equine encephalitis virus (VEE) has adjuvant characteristics in mice and guinea pigs. Infection of mice with VEE prevents the induction of tolerance to human gamma globulin and converts a tolerance-inducing stimulus into an immunity inducing stimulus.

However, unlike, LDV, which have been shown to inhibit skin allograft rejection and the graft vs host reaction, VEE has no effect on the survival of skin allografts. Thus, these virus infections have different effects on cell mediated immune reactions.

It has recently been reported that there is a relative inability to induce immunological tolerance in adult NZB mice with protein antigens. It should be noted that a virus-like agent has been implicated in the etiology of the autoimmune hemolytic anemia of NZB mice. It has been speculated that the viral agent in NZB mice may be acting as an immunological adjuvant leading to the production of antibodies against the hosts' own tissue antigens. The ability of LDV and VEE to act as adjuvants may thus lead to useful models in studying the possible role of virus infections in autoimmune phenomena. Furthermore, the wide range of host susceptibility to VEE infection permits studies on the effects of this virus on immune responses in many animal species.

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 in 1966. Dr. R. Snyderman: Invited participant to the Third International Congress of Allergy and Anaphylaxis, Toronto, Ontario, Canada, October 1968. Drs. S. E. Mergenhagen and T. Tempel: Guest lecturers at the Walter Reed Institute of Dental Research Course in Periodontics, February 1969.

Publications:

1. 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. 101: 301-307, 1968.

2. Howard, R. J., Landon, J. C., Dougherty, S. F., Notkins, A. L. and Mergenhagen, S. E.: Induction of tolerance in immunologically competent primates. J. Immunol. 102: 266-268, 1969.

3. Howard, R. J., Notkins, A. L. and Mergenhagen, S. E.: Inhibition of cellular immune reactions in mice infected with lactic dehydrogenase virus. Nature 221: 873-874, 1969.

4. Howard, R. J., Mergenhagen, S. E., Notkins, A. L. and Dougherty, S. F.: Inhibition of cellular immunity and enhancement of humoral antibody information in mice infected with the lactic dehydrogenase virus. Proc. Second International Congress of the Transplantation Society. In press, 1969.

5. Gewurz, H., Pickering, R. J., Christian, C. L., Snyderman, R., Mergenhagen, S. E. and Good, R. A.: Decreased C'1q protein concentration and agglutinating activity in agammaglobulinemia syndromes: an inborn error reflected in the complement system. Clin. Exp. Immunol. 3: 437-445, 1968.

6. Gewurz, H., Pickering, R. J., Mergenhagen, S. E. and Good, R. A.: Contrasting complement profiles in systemic lupus erythematosus, acute glomerulonephritis and hypocomplementemic chronic glomerulonephritis: clinical and experimental correlations. Int. Archives of Allergy and Applied Immunology. 34: 557-570, 1968.

7. Gewurz, H., Shin, H. S. and Mergenhagen, S. E.: Interactions of the complement system with endotoxic lipopolysaccharide: consumption of each of the six terminal complement components. J. Exp. Med. 128: 1049-1057, 1968.

8. Gewurz, H., Shin, H. S., Pickering, R. J., Snyderman, R., Lichtenstein, L., Good, R. A. and Mergenhagen, S. E.: Interactions of the complement system with endotoxic lipopolysaccharide: complement-membrane interactions and endotoxin-induced inflammation. In Cellular Recognition. Ed. by R. T. Smith, R. A. Good and P. A. Miescher, Appleton-Century-Crofts, New York, in press, 1969.

9. Lichtenstein, L. M., Gewurz, H., Adkinson, S. F., Shin, H. S. and Mergenhagen, S. E.: Interactions of the complement system with endotoxic lipopolysaccharide: Generation of an anaphylatoxin. Immunology, in press, 1969.

10. Snyderman, R., Gewurz, H. and Mergenhagen, S. E.: Interactions of the complement system with endotoxic lipopolysaccharide: Generation of a factor chemotactic for polymorphonuclear leukocytes. J. Exp. Med. 128: 259-275, 1968.

11. Jensen, J., Snyderman, R. and Mergenhagen, S. E.: Chemotactic activity, a property of guinea pig C'5-anaphylotoxin. Third International Congress of Allergy and Anaphylaxis, in press, 1969.

12. Snyderman, R., Shin, H. S., Phillips, J. K., Gewurz, H. and Mergenhagen, S. E.: A neutrophil chemotactic factor derived from C'5 upon interaction of guinea pig serum with endotoxin. J. Exp. Med. in press, 1969.

13. Shin, H. S., Snyderman, R., Friedman, E., Mellors, A. and Mayer, M. M.: Chemotactic and anaphylotoxic fragment cleaved from the fifth component of guinea pig complement. Science, 162: 361-363, 1968.

14. Mergenhagen, S. E., Snyderman, R., Gewurz, H. and Shin, H. S.: Significance of complement to the mechanism of action of endotoxin. Current Topics in Microbiology and Immunology, in press, 1969.

Serial No. NIDR-16 (61)
1. Microbiology
2. Microbial Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Studies on the Regulation of Carbohydrate Fermentations and Lactic Acid Production in Microorganisms

Previous Serial Number: NIDR-18

Principal Investigator: Dr. C. L. Wittenberger

Other Investigators: Dr. A. T. Brown
Dr. F. Gasser (Visiting Scientist)

Cooperating Units: None

Man Years:

Total:	6 1/4
Professional:	3
Other:	3 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. This report is a summary of studies that were oriented specifically toward resolving certain degradative metabolic pathways that operate in various homo- and heterofermetative lactic acid bacteria. Factors involved in the regulation of these pathways were also examined.

Methods Employed:

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

Major Findings:

Streptococcus faecalis

Regulation of function of enzymes involved in carbohydrate fermentation. The lactate dehydrogenase (LDH) from S. faecalis requires fructose-1,6-diphosphate (FDP) for activation. Previous work suggested that the activation involved an allosteric transition from a constrained configuration to one in which the coenzyme and substrate binding sites were more exposed. Results from additional kinetic and physical studies are in accord with this hypothesis. The transition in state appears to involve a change in the tertiary structure of the LDH and not a change in its quaternary structure. The enzyme has a molecular weight (as determined by sucrose density gradient centrifugation)of about 68,000 both in the presence and the absence of FDP.

The S. faecalis LDH appears to be an oligomer, however. It has been possible to treat the enzyme with guanidine hydrochloride in such a way as to cause a loss of about 80% of its catalytic activity and then,under appropriate conditions, to demonstrate a time-dependent reactivation. The maximum activity recovered to date has been about 30% of that lost. These results suggest that the enzyme is composed of two or more subunits which dissociate in guanidine hydrochloride and which can reassociate under appropriate conditions to form a catalytically active unit.

FDP appears to be a key compound in the regulation of catabolic pathways in S. faecalis. In addition to its role as an activator for the LDH, this compound has been shown to be a potent and specific inhibitor of the enzyme 6-phosphogluconate dehydrogenase (6PGD). It exerts its maximum inhibitory effect on 6PGD at a concentration that activates the LDH maximally. Thus, under conditions where the cell requires energy, carbon flow may be directed through the energy-liberating Embden-Meyerhof-Parnas pathway and away from the nonenergy-liberating hexose monophosphate pathway by maintenance of a critical pool level of FDP. Conversely, if the cell is rich in energy but deficient in NADPH required for biosynthesis, carbon flow may be redirected through the hexosemonophosphate pathway and away from the Embden-Meyerhof-Parnas pathway by lowering the endogenous pool of FDP.

It is considered highly significant that kinetic studies on the inhibition of 6PGD by FDP have shown that the enzyme can not be completely inhibited even at very high levels of FDP. In fact, the greatest degree of inhibition that can be achieved in vitro is only about 80% of the maximum rate. This means that even under the most stringent demands for energy, when the cell would be

expected to have a high endogenous pool of FDP, a limited amount of glucose carbon could pass through the hexosemonophosphate pathway to supply the cell with pentose for DNA and RNA synthesis as well as some NADPH for other essential biosynthetic processes.

The regulation of 6 PGD activity by FDP appears to be a general phenomenon. This glycolytic intermediate has been shown to inhibit 6-phosphogluconate dehydrogenases from a wide variety of microorganisms and from mammalian tissue as well.

Gluconate fermentation: The ability of S. faecalis to grow anaerobically on gluconate is something of an enigma. A substantial proportion of the gluconate degraded has been shown by C^{14} studies to pass through the hexosemonophosphate pathway. The first two enzymes of this pathway have been found to be NADP-specific dehydrogenases. However, there appeared to be no way in which the NADPH coenzymes could be reoxidized since the major enzyme responsible for reoxidizing reduced pyridine nucleotides in this organism is an NADH-specific lactate dehydrogenase. Moreover, cell-free extracts of the organism were devoid of transhydrogenase activity. This problem was resolved by demonstrating that S. faecalis produced substantial amounts of ethanol during anaerobic growth on gluconate, and that the ethanol dehydrogenase was an NADPH-specific enzyme. This enzyme was inducible, appearing only during anaerobic growth on gluconate, and its biosynthesis was repressed when glucose or pyruvate was included in the growth medium.

Mannitol fermentation: It is well known that S. faecalis can grow anaerobically at the expense of mannitol or sorbitol. However, the disposition of the extra pair of reducing equivalents (compared to glucose) generated in the degradation of these compounds could not be accounted for in a theoretical homolactic fermentation. A study of the polyalcohol fermentations has revealed that the extra reducing equivalents generated in the conversion of these compounds to fructose are coupled to ethanol formation by an NAD-specific ethanol dehydrogenase. The enzyme is inducible and its biosynthesis is repressed by glucose. It differs from the ethanol dehydrogenase formed during growth of the organism on gluconate in that the "gluconate-enzyme" is specific for NADP. Moreover, it exhibits different kinetic properties from an NAD-linked ethanol dehydrogenase that the organism forms during growth on malate.

Streptococcus mutans: Specific biochemical steps involved in the fermentation of various compounds have been examined in a number of strains of cariogenic streptococci. These strains have included organisms of hamster, rat and human origin. The following summary deals primarily with results obtained from a human strain. Streptococcus mutans (NTCC 10449).

Polyalcohol fermentation: S. mutans forms an inducible fructose-6-phosphate reductase(s) when grown on mannitol or sorbitol as well as an inducible ethanol dehydrogenase. Both the reductase(s) and ethanol dehydrogenase are NAD-specific enzymes and in this respect resemble S. faecalis. The organism produces no ethanol from either mannitol or sorbitol when glucose or fructose is included in the growth medium.

Gluconate Fermentation: S. mutans is unable to decompose gluconate. This is true for all of the strains so far tested and represents a heretofore unreported phenotypic characteristic of this group of organisms. In vitro studies have indicated that this deficiency is due to the fact that these organisms do not possess the first two enzymes required for the dissimilation of gluconate through the hexosemonophosphate pathway. Their inability to decompose gluconate by this pathway in vivo has been confirmed by means of C^{14}-gluconate studies with whole cells.

Fermentation of 3-Carbon Compounds: Another unreported phenotypic characteristic of S. mutans strains is their inability to ferment pyruvate or glycerol. Upon analyzing extracts of these organisms for various Embden-Myerehof-Parnas enzymes, it was found that strain 10449 lacks fructose diphosphatase. This is a key enzyme involved in the synthesis of 6-carbon compounds from 3-carbon precursors, and its absence may well explain the inability of this organism to grow on pyruvate or glycerol.

Lactate Dehydrogenase: The LDH from S. mutans, like the LDH from S. faecalis, requires FDP for catalytic activity. The enzyme from S. mutans is quite different from the S. faecalis LDH, however, and can easily be distinguished from it on the basis of several kinetic and physical properties. The S. mutans LDH, for example, requires about 5 mM FDP for complete activation, while the S. faecalis enzyme is maximally activated at an FDP concentration of 0.04 mM. Moreover, the S. mutans LDH is normally very heat-labile and is protected from thermal inactivation by FDP, whereas, the S. faecalis enzyme is normally heat-stable and is very susceptible to thermal inactivation in the presence of FDP. The properties of the S. mutans LDH appear to be unique among several other members of the genus Streptococcus so far examined and it seems likely that they may be a useful taxonomic tool. Also of importance is the possibility that these properties may serve as a diagnostic aid for distinguishing cariogenic from non-cariogenic streptococci. A more extensive comparative study of lactate dehydrogenases from other streptococci is anticipated as a means of assessing the validity of this possibility.

Studies with members of the genus Lactobacillus:

Physiology and Biochemistry. A number of species representing both homo- and heterofermentative lactobacilli have been examined for the presence and cofactor specificity of certain key dehydrogenases involved in carbohydrate fermentation. Both groups of organisms possess dehydrogenases of the hexosemonophosphate pathway, although higher levels of these enzymes are found in the heterofermentative group. In general these enzymes function with either NAD or NADP in the homofermentative organisms whereas they function with both coenzyme in the heterofermentative group. The heterofermentative organisms possess an ethanol dehydrogenase while the homofermentative group appear to be devoid of this enzyme.

Taxonomy: Work has been initiated that is directed toward establishing new criteria by which existing species within the heterofermentative lactobacilli may be identified and distinguished from one another. On the basis of patterns of electrophoretic mobility of lactate dehydrogenases from a limited number of strains, prior work suggested that there were five groups of heterofermentative organisms. Some 30 new strains have been analyzed by this technique and the results confirm this grouping.

Significance to Dental Research:

It is anticipated that information derived from these studies on biochemical control methanisms 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 fermentation pathways and 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 Project:

More comprehensive studies are planned on the mechanism of activation of the lactate dehydrogenase from S. mutans 10449 by fructose-1, 6-diphosphate. This enzyme is clearly different from the LDH's found in Group D streptococci or S. lactis and a detailed analysis of its kinetic and physical properties should yield information that will be useful in distinguishing this group of organisms from other streptococci. A further analysis of various fermentation pathways is also contemplated with a view toward defining more completely the metabolic capabilities of this group of organisms. Particular emphasis will be given to pathways involved in 6-carbon metabolism and factors which function to regulate these pathways.

Studies on gluconate metabolism in S. faecalis will be continued. The means by which an intermediate from glycolysis (FDP) inhibits the activity of a hexose monophosphate pathway enzyme (6-phosphogluconate dehydrogenase) will be assessed in more detail and other enzymes of this pathway will be examined as possible sites for metabolic control. Efforts are also contemplated to resolve the biochemical mechanism by which this organism regulates the cellular pool level of FDP.

The ethanol dehydrogenase formed by S. faecalis during growth on gluconate will be examined and its properties compared with the enzyme present in mannitol-, sorbitol-, or malate-grown cells.

Studies on the five groups of heterofermentative lactobacilli will be continued and expanded. A comparison of the electrophoretic migration of glucose-6-phosphate dehydrogenases and ethanol dehydrogenases from representative strains of each group will be made.

Part B:

Publications:

Wittenberger, C. L., and Angelo, N.: Mechanism of activation of lactate dehydrogenase from Streptococcus faecium by fructose-1, 6-diphosphate. Bacteriol. Proc. p. 118, 1969.

Serial No. NIDR - 17 (66)
1. Microbiology
2. Microbial Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Systematic Microbiological Taxonomic Studies

Previous Serial Number: NIDR-13

Principal Investigator: Morrison Rogosa

Other Investigators: Minimal assistance on some phases of this project provided by investigators designated in text.

Cooperating Units: Division of Computer Research & Technology, NIH; 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 system 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 cultural, 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 study of the anaerobic gram negative organisms described in last year's report was completed and thus was described as a new genus Acidaminococcus gen. nov., capable of fermenting amino acids, particularly glutamic acid, to butyric and acetic acids, and CO_2. Statements in last year's report identifying the new organisms with Peptococcus are incorrect. A manuscript describing the new genus and its relation to Veillonella and Peptococcus is being published in the J. Bacteriol. 97: May 1969.

The studies on the utilization of malate are continuing. Comprehensive surveys showed that lactobacilli can not utilize malate for growth but Lactobacillus casei and L. plantarum degrade malate only after growth has occurred at the expense of a substrate other than malate. In addition, lactobacilli can not grow on pyruvate although pyruvate is obviously metabolized.

Dr. London is studying the malic enzyme in streptococci and is also making a major contribution to the study of malic dissimilation in lactobacilli.

Growth studies with gluconate as substrate for the heterfermentative and Streptobacterium groups of lactobacilli have been completed.

Although this extensive material is ready for publication, we prefer to wait for the completion of Dr. Albert Brown's enzymological studies so that an integrated series of papers can be published simultaneously.

Our gas chromatographic technique (Applied Microbiology 16: 285-290, 1968) has been applied to the sensitive and quantitative analysis of ethanol and acetate in many samples from a number of studies in the Laboratory of Microbiology.

In cooperative work with Dr. Francis Gasser, cell preparations were made for guanine and cytosine (GC) molar percent of the DNA of important type strains of lactobacilli. Also, complete phenotypic reactions were determined for lactobacillus strains where lactic dehydrogenase (LDH) electrophoretic mobilities have already been done by Dr. Gasser. Thus, we have correlative genotypic, phenotypic, and enzymatic data for these important strains.

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 were made for their improved definition in the forthcoming edition of Bergey's Manual. This meeting was mandatory. A second meeting was held in Canterbury, England, on July 1-5, 1968.

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. A manuscript for Bacteriological Reviews is being prepared, illustrating the information which can be stored, coded, retrieved, assessment techniques, etc.

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 of 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.

4. A number of manuscripts for Bergey's Manual have already been written but about half the remaining manuscripts are to be finished by October 1, 1969. All of the manuscripts will be submitted in a single package.

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 American 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 Trust to rewrite the description of the genera Veillonella, Lactobacillus, Lachnospira, Peptococcus, Bifidobacterium, Ruminococcus, Peptostreptococcus, Acidominococcus.

Appointed to the Board of Editors of Bacteriological Reviews.

Publications:

Rogosa, M.: Acidominococcus gen. nov., Acidominococcus fermentans sp. nov. anaerobic gram-negative diplococci using amino acids as the sole energy source for growth. J. Bacteriol. 87: 1969.

Serial No. NIDR - 18 (66)
1. Microbiology
2. Microbial Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

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

Previous Serial Number: NIDR-13

Principal Investigator: Dr. J. P. London

Other Investigators: None

Cooperating Units: None

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 group D and cariogenic streptococci as well as other microorganisms. This entails physiological studies aimed at defining the general growth conditions responsible for induction and repression of catabolic enzymes and a biochemically-oriented characterization of those enzymes.

Methods Employed:

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

Major Findings:

The ability of the lactic acid bacteria to grow at the expense of a variety of substrates including carbohydrates, polyalcohols and mono- and dicarboxylic acids is a well documented but unappreciated trait of this group of microorganisms. Many enzymes which catalyze the dissimilation of these organic substrates are apparently inducible, and at the present time virtually nothing is known about the regulation of synthesis or function of this class of enzymes. Two types of inducible dehydrogenases are presently under investigation.

1. Malic enzyme. 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 differed 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 the addition of relatively low concentrations of glucose (below 0.1%) to a culture growing at the expense of L(+) malate. However, despite the synthesis and accumulation of malic enzyme in growing cultures containing malate and glucose, the former was not utilized until the exogenous supply of glucose had disappeared. The preferential dissimilation of glucose in the absence of catabolite repression suggested that the synthesized malic enzyme was nonfunctional during glucose utilization.

The biochemical and physical properties of the malic enzyme were described in last year's report. Two intermediate products of the Embden-Meyerhof-Parnas pathway, fructose-1, 6-diphosphate and 3-phosphoglycerate, and 6-phospho-gluconate, an intermediate product of the hexose monophosphate pathway, were shown to be allosteric inhibitors of the malic enzyme. The nonfunctional state of the malic enzyme during growth in the presence of low levels of glucose was attributed to the accumulation of these inhibitory intermediate products. In addition, an allosteric inhibition was demonstrated with nucleoside triphosphates; ATP was the most efficient inhibitor of these tested.

Recently it was found that increasing the concentration of glucose in malate-containing media to 0.2% or greater resulted in a marked repression of the malic enzyme. Under these conditions of growth the apparent "diauxic effect" was not observed. Although cell suspensions prepared from cultures grown under these conditions contained reduced levels of the malic enzyme, they were unable to metabolize malate following the removal of glucose and its intermediate products by repeated washings. Hence, the inability of resting cell suspensions to metabolize malate at a significant rate could not be attributed to feedback inhibition. Permeation studies using C^{14}-malate demonstrated that a malate permease was induced concomitant with the induction

of the malic enzyme and that the glucose effect, in fact, represented a catabolite repression of the malate permease. The permease once formed was not inhibited by glucose or intermediate products of glycolysis. However, inhibition of permease synthesis apparently prevented the entry and/or accumulation of sufficient amounts of malate to act as inducer for the malic enzyme. Hence, a coincidental repression of synthesis of two induced enzymes to be operative here. This novel form of regulation may be widespread and other inducible enzyme systems among the lactic acid bacteria should be investigated.

2. Polyalcohol Dehydrogenases. A physiological and biochemical characterization of three polyalcohol dehydrogenases, sorbitol, mannitol and glycerol dehydrogenase, has been initiated. These two former enzymes are inducible systems in the group D and cariogenic streptococci. In both instances the enzymes are NAD-specific and require the appropriate phosphorylated substrates, mannitol-1-phosphate and sorbitol-6-phosphate.

Preliminary purification of these enzymes has been attempted. Both are relatively stable to the usual chemical treatments and the purification of these enzymes should not prove difficult.

The current study will eventually be extended to other inducible systems responsible for the dissimilation of sucrose and lactose. Information pertinent to the regulation of these systems should have immediate application to the problems of dental caries.

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. The investigation of the physiological and biochemical properties of the streptococcal malic enzyme is being extended to the homo- and heterofermentative lactobacilli. This work, performed in collaboration with Mr. M. Rogosa, will attempt to determine the degree of evolutionary relatedness between the two groups of lactic acid bacteria. Subsequently, the relationships within the groups will be probed as well. Biochemical and immunological studies of this nature are imperative for developing meaningful taxonomic relationships among members of this group of organisms.

2. The role of catabolite repression of specific substrate permeases will be studied in other inducible enzyme systems, i.e., glycerol, sorbitol, and mannitol dehydrogenases. Procedures similar to those described for the malate permease will be modified to accommodate the present systems for further studies.

3. A physiologically and biochemically oriented study of polyol dehydrogenases is underway and will be continued. After characterizing the processes of induction and repression of these enzyme systems in growing cultures, the enzymes will be purified and prepared for immunological studies. This will permit a direct assessment of the relationships between the Group D and cariogenic streptococci. At the same time, enzymes from both groups of organisms will be studied to determine the degree of similarity of their chemical and physical properties. In addition to providing much needed physiological information about the cariogenic streptococci, this work will contribute data which will aid in establishing an accurate taxonomic position for the Streptococcus mutans group within the genus.

Part B:

Publications:

1. London, J., and Meyer, E. Y. Malate utilization by a group D Streptococcus. Physiological properties and purification of an inducible malic enzyme. J. Bacteriol. In press, 1969.

2. London, J., and Meyer, E. Y. Malate utilization by a group D Streptococcus. Evidence for allosteric inhibition of an indicible malic enzyme by ATP and glycolytic intermediate products. Biochem. Biophys. Acta. 178: 205-212, 1969.

Serial No. NIDR - 19 (67)
1. Microbiology
2. Virology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Recurrent and Persistent Viral Infections

Previous Serial Number: NIDR-15

Principal Investigator: Dr. A. L. Notkins

Other Investigators: Mr. W. K. Ashe
Dr. C. A. Daniels
Dr. N. L. Levy

Cooperating Units: National Cancer Institute

Man Years:

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

Project Description:

Objectives:

To study 1) factors involved in recurrent and persistent virus infections, 2) the mechanism of virus neutralization and 3) the effect of virus infections on the functional capacity of the immune system.

Methods Employed:

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

Major Findings:

Neutralization of Sensitized Herpes-Simplex Virus by Complement. Previously we showed that the interaction of virus with antiviral antibody results in the formation of an infectious virus-antibody complex (sensitized virus) and that this complex could be neutralized by anti-γ-globulin, antiviral antibody or complement. Virtually nothing was known, however, about the mechanism of

Part A (continued)

neutralization of sensitized virus by complement. Experiments performed over the past 12 months provided new insight into the mechanism of action of complement. Using the purified components of complement recently isolated by Drs. Boros and Rapp of the National Cancer Institute, we found that herpes simplex virus (HSV) which had been sensitized with anti-HSV (IgM) antibody could be neutralized under the appropriate conditions by the fourth component of complement and that the neutralization was enhanced by the addition of the second and third components of complement. These findings represent a hitherto unreported biological function for these components of complement and support the hypothesis that complement neutralizes sensitized virus by the piling up of the components on the surface of the virion. Our data also suggests that in vivo, the first and fourth components of complement may be the only components needed for virus neutralization. The ability of the purified components of complement to neutralize virus sensitized with other classes of immunoglobulins is under study.

Neutralization of Sensitized Herpes Simplex Virus by Anti-γ-Globulin. As indicated last year, the interaction between anti-γ-globulin and the γ-globulin on the sensitized virion makes it possible to use sensitized virus as a highly specific and sensitive indicator system for detecting and characterizing antiviral immunoglobulins. This year we showed that the anti-γ-globulin technique could be used to 1) detect univalent fragments of antiviral antibody and 2) to recognize allotypic differences between immunoglobulin molecules. In addition, we showed for the first time that univalent fragments of anti-HSV γ-globulin could neutralize and/or sensitize HSV. Furthermore, the sensitized virus could be neutralized by either undigested anti-γ-globulin or the univalent fragments of anti-γ-globulin. The specificity involved in the neutralization of sensitized virus by anti-γ-globulin was shown by the fact that virus which had been sensitized with undigested or papain-derived univalent fragments of anti-HSV γ-globulin on one allotype was neutralized by anti-γ-globulin directed against the allotype but not by anti-γ-globulin directed against a different allotype.

The anti-γ-globulin technique has also led to the development of a new technique for measuring antibody to viral immunoglobulins. Antibody to human-IgG was measured by its ability to neutralize virus which had been sensitized with human anti-HSV IgG. A number of the quantitative and qualitative factors involved in this system were elucidated. A comparison of the sensitized virus technique with the red cell agglutination technique (which has been regarded as one of the most sensitive methods for measuring antibody) showed that the former technique was as sensitive and in certain cases more sensitive than the later technique. In addition, sensitization of the virus makes it possible to separate antiviral

immunoglobulins from the rest of the immunoglobulin pool and to study the interaction of specific anti-immunoglobulins with the antiviral immunoglobulins.

Neutralization of Sensitized Herpes Simplex Virus by Univalent Antiviral Antibody Fragments - Chronic Viral Infections. Previously we reported that sensitized virus was neutralized at a slower rate by antiviral antibody than unsensitized virus. These and other studies suggested that sensitization might in part be responsible for the chronic nature of certain viral infections. Studies initiated last year on the kinetics of neutralization of sensitized virus with univalent fragment of antiviral antibody were completed and published. We found that sensitization of HSV with undigested anti-HSV reduced the neutralization rate constant by as much as 71% when tested with undigested anti-HSV, but resulted in only a 5% reduction in the neutralization rate constant when tested with univalent fragments of anti-HSV. These observations suggest that the smaller univalent fragments, in contrast to the larger undigested divalent antibody molecules, were more readily able to reach antigenic sites on the surface of the sensitized virion. These findings suggest that the univalent antibody fragments might prove useful in the neutralization of virus which is highly sensitized and relatively resistant to neutralization by undigested antiviral antibody.

Pertinent to the mechanism of virus neutralization was the demonstration that highly sensitized HSV penetrated mammalian cells at a significantly slower rate than unsensitized virus. This information is important to workers in the field of viral immunology for the proper interpretation of certain types of neutralization data. In connection with the biological significance of sensitized virus it should be mentioned that Dr. Frank Dixon at the Scripps Foundation carried out experiments which suggest that, in vivo, sensitized virus (lymphocytic choriomeningitis virus) might be responsible for certain chronic diseases such as glomerulonephritis.

Physical and Chemical Studies on Sensitized Virus. Over the past 12 months a number of new techniques were set up in our laboratory to study some of the physical and chemical properties of sensitized virus. We have successfully labeled HSV with H^3 thymidine and purified the labeled virus by sucrose gradient sedimentation. We are now in the process of studying the interaction of the anti-HSV with the labeled virus. Efforts are also being made to separate sensitized virus from unsensitized virus by sucrose gradient sedimentation. In other experiments we are attempting to label antiviral antibody with I^{125}. In collaboration with Dr. Howard Bladen of the Laboratory of Histology and Pathology we are studying by electron microscopy the interaction of ferritin labeled anti-γ-globulin with sensitized virus. Because of its small size and

Part A (continued)

stability, polyoma virus offers certain advantages over HSV for physical and chemical studies. We have recently set up the assay for polyoma virus in our laboratory and we are attempting to sensitize the virus with anti-polyoma antibody. There are no reports, thus far, on the sensitization of tumor viruses. Studies on the physical and chemical properties of another virus of the oral cavity, the rat submaxillary gland virus, were completed and published.

Attempts to Recover "Latent" Viruses by Cell Fusion. Since HSV produces recurrent lesions in certain individuals it is widely thought that this virus exists in a "latent" state. If this were the case it might be possible to recover the "latent" virus from cells suspected of harboring the virus by fusing these cells with rabbit kidney cells which are highly susceptible to infection with HSV. This past year we have set up the technique for fusing cells by employing inactivated Sendai virus as the fusing agent. The fusion technique is also being used to study the transfer of information from macrophages to lymphocytes in hopes of learning more about antibody production.

Effect of Virus Infection on the Functional Capacity of the Immune System. We have continued our studies in collaboration with the Immunology Section on the effect of virus infections of the functional capacity of the immune system. We found that infection of adult mice with lactic dehydrogenase virus (LDV) significantly inhibited the rejection of skin allografts. In addition, infection of newborn mice with LDV depressed the graft-versus-host reaction. These studies together with our earlier findings that LDV can enhance humoral antibody production and prevent the development of immunological tolerance indicates that viruses can profoundly alter the immune system. We are presently writing a review on this subject entitled "The Effect of Virus Infections on the Functional Capacity of the Immune System."

Immunological Tolerance in Primates. Lastly, in collaboration with the Immunology Section, we succeeded in making adult monkeys tolerant to human γ-globulin. This is the first time that immunologically mature primates have been made tolerant to a soluble antigen. These findings encourage us to believe that it might be possible to make mature primates tolerant to allografts by use of soluble transplantation antigens.

Significance to Dental Research:

Our findings provide new insight into the mechanism of neutralization of sensitized herpes simplex virus (HSV) by complement and have revealed a hitherto unreported biological function for the fourth component of complement.

Part A (continued)

The demonstration that sensitized HSV could be neutralized by univalent fragments of anti-HSV suggest that these fragments might prove useful in the neutralization of virus which is highly sensitized and relatively resistant to neutralization by undigested divalent antiviral antibody.

The demonstration that virus infections can profoundly alter the functional capacity of the immune system might be important in terms of the effect that virus infections might have on local immunity in the oral cavity.

Finally, we showed for the first time that immunologically mature primates (monkeys) could be made tolerant to a soluble antigen (human γ-globulin). These findings encourage us to believe that it might be possible to make mature primates tolerant to allografts and in particular to the transplantation of oral tissues.

Proposed Course of Project:

Present experiments are concerned with 1) the mechanism of neutralization of sensitized virus by complement, anti-γ-globulin antiviral antibody, and rheumatoid factor; 2) studies on the physical-chemical properties of sensitized virus; 3) the role that sensitization might have on the persistence of viral infections; 4) the effect that sensitized virus might have on the development of "chronic" and "autoimmune" diseases; 5) the effect of virus infections on the functional capacity of the immune system; 6) the recovery of "latent" viruses by the cell fusion technique; 7) the transfer of viral and immunological information from one cell to another; and 8) the susceptibility of cells from individuals with different genetic diseases to infection with HSV.

Part B:

Publications:

1. Ashe, W. K., Mage, M., Mage, R., and Notkins, A. L.: Neutralization and sensitization of herpes simplex virus with antibody fragments from rabbits of different allotypes. J. Immunol. 101: 500-504, 1968.

2. Ashe, W. K.: Properties of the rat submaxillary gland virus haemagglutinin and antihaemagglutinin and their incidence in apparently healthy gnotobiotic and conventional rats. J. Gen. Virol. 4: 1-7, 1969.

3. Ashe W. K., Mage, M., and Notkins, A L.: Kinetic of neutralization of sensitized herpes simplex virus with antibody fragments. Virology, 37: 290-293, 1969.

Part B (continued)

4. Howard, R. J., Landon, J. C., Dougherty, S. F., Notkins, A. L., and Mergenhagen, S. E.: Induction of tolerance in immunologically competent primates. J. Immunol. 102: 266-268, 1969.

5. Howard, R. J., Notkins, A. L., and Mergenhagen, S. E.: Inhibition of cellular immune reactions in mice infected with lactic dehydrogenase virus. Nature 221: 873-874, 1969.

6. Howard, R. J., Mergenhagen, S. E., Notkins, A. L., and Dougherty, S. F.: Inhibition of cellular immunity and enhancement of humoral antibody formation in mice infected with lactic dehydrogenase virus. Transplantation Proceedings 1: 586-588 1969.

7. Notkins, A. L.: Neutralization of sensitized virus by anti-γ-globulin. Perspectives in Virology 6: 189-192, 1968.

Annual Report of the Human Genetics Branch
National Institute of Dental Research
Summary Statement

The activities of the Human Genetics Branch are encompassed by two sections; Developmental Genetics and Population Genetics. The interests and activities of the two groups overlap and compliment each other in many areas. Functionally the Developmental Genetics Section includes all activities involving laboratory procedures while the Population Genetics Section is involved in mathematical, statistical and epidemiological activities.

DEVELOPMENTAL GENETICS

The investigations of the Developmental Genetics Section concern the action of genes on oral and dental traits at several levels of detail. Gene control of the histological structure of the enamel, control of the morphological pattern of the whole tooth and the interrelations among teeth comprise one aspect of the program. The interaction between specific genes and the teratogenic action of vitamin D are being investigated in collaboration with other NIDR investigators. Evidence for gene action in the mechanisms of taste and smell are being sought, while another part of the program is involved with the study of variation in the constituents of human saliva. A specific hereditary syndrome involving abnormal development or function of the neural crest with pleiotropic effects is also being investigated. In all aspects, the basic design is to investigate the morphology and physiology of individuals with genetic abnormalities. Comparisons to normal are made in form and function and in the developmental processes.

Dental Anomalies in Rodents

A recessive mutant has occurred in the laboratory mouse which causes abnormal hair and "soft" teeth. This mutant stock has been obtained from Los Alamos and a colony has been established. Histological study of the defective homozygotes compared with heterozygote and normal homozygote animals from the same line are in progress. The nature of the biochemical defect in enamel production will be investigated using special staining and biochemical procedures. Several other mutant lines have been studied because of defects in neural crest derivatives. Two of three lines show no dental abnormality. The third line is being investigated in embryological material because of lethal effects before eruption. Radioisotope study of the mitotic activity of hair follicles in the "downless" mouse embryo are being carried out to attempt to localize the time of arrest in the development of these mice which have defects of both hair and teeth.

Morphological abnormalities of the molars of the rice rat have been observed by James Shaw at Harvard. He has cross mated many of the animals with fused and supernumerary teeth. Genetic analysis of his data suggests a major gene effect modified by genetic background and/or environment. Further genetic and embryological studies of this morphological abnormality are planned when our colony is established.

Hypervitaminosis D

Hypervitaminosis D in the pregnant rabbit has been reported to produce anomalies of the orofacial complex, and cardiovascular abnormalities in the offspring. Whether the variation in the teratogenic effect of standard dosage is of genetic origin is not known. The appearance of many of the offspring of treated mothers resembles hereditary buphathalmos which is known to occur in the NIH rabbit colony. The general metabolic effects of the vitamin D treatment and the pedigree distribution of the dose response is being investigated. Further study of the quantitative aspects of calcium metabolism in treated and control animals is directed at the relation of maternal levels of calcium and phosphorous to the teratogenic effect.

Deafness

Albinoid pigmentation is associated with sensory defects and malformations in many species. Because of the known contribution of the neural crest to the development of pigment and the sensory nervous system, it appears probable that the primary defect is in the behavior or development of the neural crest. The most extensive histological study of the ear defect in deaf white animals has been in the cat. A colony of deaf white cats, developed in collaboration with NINDS, is being subjected to intensive genetic neurophysiological and histological study in order to localize the defect and better characterize its development. This program will terminate next year.

Taste and Smell Perception

The chemical senses in man are peculiarly stomal. Genetic variations in the abilities of taste and smell are almost totally unexplored, although variation among individuals is well recognized. Genetic variations, if discrete, would be valuable as indicators of the mechanisms of the taste or smell response and also as genetic markers for the study of human families and populations.

Preliminary studies suggest that the threshold of sensitivity of the normal individual is the result of several factors, including one of general sensitivity. Despite influence of the general factor, specific lack of sensitivity does occur. Attempts are being made to screen adult human populations for specific lack of sensitivity with the intent of doing family studies on the abnormal individuals. The data will be processed to make correction for the general factor of sensitivity. Extreme variants of general sensitivity will also be followed up in family studies.

Saliva Studies

Variations in the proteins of human saliva have been observed using discontinuous acrylamide electrophoresis. Some variations appear to show familial segregation. The aspects of quantitative variability are being examined for heritability using identical and fraternal twins.

POPULATION STUDIES

Malocclusion and Caries

The major effort in this area involves the study being conducted in collaboration with the University of Hawaii. The objective of this study is to describe racial variation in dental traits and specifically to assess the affects of racial intermixture. Examinations have been completed on approximately 18,000 children ages 12 to 17 and analysis has begun. Considerable racial variation has been noted in dental development as well as various dental diseases. Preliminary analysis indicates no detectable affect of racial hybridization in malocclusion. However, certain environmental factors seem to play a role. Dental caries rates are also quite different between races but there is no evidence that genetic factors are involved.

Dental Variability in Man

Studies continue to be directed at assessing factors (genetic and environmental) responsible for morphological and developmental variability in the dentition. Dental observations are being made on various Indian groups including Pima, Papago, Zuni and other Pueblo Indians. These data are to be used in conjunction with blood group data from the same tribes in order to study how closely dental morphological variation corresponds to genetic variation as determined on the basis of known genetic polymorphisms.

Other studies relate the effects of various prenatal factors on dental variability. One significant finding in this regard is that of a positive correlation between birth weight and dental development at 6 to 12 years of age. Although other explanations exist, this finding suggests that prenatal factors responsible for low birth weight also act to retard dental development and that this effect continues to late childhood. Correlation has been demonstrated between the degree of dental asymmetry and environmental stress to which a population is subjected. This again emphasizes the importance of environmental factors in dental development.

Genetic Variation in Human Populations

Activities during the last year have been directed toward the study of the Papago Indians. Significant local genetic differentiation has been demonstrated, related in large part to geographic isolation of subgroups within the tribe. Genetic differences between these subgroups of the Papago

are greater than the overall differences observed between the Papago and Pima (a closely related tribe). Studies of a more theoretical nature have been directed at the study of those factors (e.g., mating pattern, selection, intermixture) affecting the genetic constitution of populations, as well as the effect of population size and age structure on birth and death rates.

Congenital Malformation in the American Indian

This study is now in its fifth and probably final year. Analysis is underway on data from approximately 45,000 Indian births. Of particular interest is the comparison of American Indian malformation experience with that of other major racial groups. Much of the analysis will be concerned with inter-tribe variation and the effects of Caucasian admixture on the total malformation rate as well as for specific malformations. In addition, many of the usual variables of epidemiologic interest such as season of birth, parental age, and parity will be examined.

Preliminary results suggest that cleft lip $\pm$ cleft palate has a frequency in the American Indian intermediate between those of other Mongoloid populations and the U. S. Caucasians. Isolated cleft palate on the other hand shows frequencies almost identical to those reported for the Japanese. Similar results have been obtained for anencephaly and spina bifida. The frequency of spina bifida in the American Indian is intermediate between Japanese and Caucasian, whereas anencephaly approximates that of the Japanese. It has also been noted that there are significant difference between tribes in the relative frequencies of cleft lip $\pm$ cleft palate compared to isolated cleft palate.

Other studies on oral clefts have involved an analysis of births for a ten year period in two states directed at the detection of temporal or spatial clustering. No such clusters have been detected.

Serial No. NIDR-20 (67)
1. Human Genetics
2. Developmental Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Developmental Processes in Genetically Controlled Traits

Previous Serial Number: NIDR-34

Principal Investigator: Dr. K. S. Brown

Other Investigators: Dr. J. A. Sofaer and Dr. D. R. Bergsma

Cooperating Units: Dr. E. L. Eagles, NINDS
Dr. J. Shaw, Department of Nutrition,
Harvard School of Dental Medicine

Man Years

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

Project Description:

Objectives:

1. To determine the nature of the genetic control of certain dental defects in rodents. These include structural defects of enamel in the mouse as well as fused and supernumerary molars in the rice rat.

2. To investigate the developmental mechanisms involved in the production of these defects.

3. To investigate the mode of action of the two genes tabby (Ta) and downless (dl) which are known to cause abnormalities of the teeth and hair in mice.

4. To determine the nature and degree of dental involvement in three mouse mutants (Sp^d, s^l, and ls) which produce abnormalities in tissues derived from the neural crest.

5. To establish methods appropriate to the analysis of dental variation in man.

6. To investigate the genetics of a syndrome of defects of pigmentation, inner ear, and eye in the white cat.

7. To examine the histology of the ear and eye of white cats in relation to the ability of the animals to hear and see.

8. To study the reproduction and survival of white cats in an isolated colony and in an open colony.

Methods Employed:

1. Experimental crossing of mice with and without the enamel defect, and analysis of rice rat breeding records supplied by Harvard School of Dental Medicine.

2. Routine histological examination of the developing teeth and dental tissues.

3. Analysis of vibrissa number in normal and tabby mice. Measurement of H^3 thymidine uptake by developing normal and downless embryos.

4. Establishment of stock colonies of the three neural crest mutants, and their investigation by gross and histological examination.

5. Review of established methods used in genetical analysis and selection of those appropriate to dental problems.

6. 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 NINDS. 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:

1. The enamel defect in mice is almost certainly caused by a single recessive gene which is also responsible for a hair defect. There is very good evidence that fused and supernumerary molars in the rice rat are produced by a single recessive gene, although this gene, if it exists, is subject to considerable modification of expression.

2. Preliminary histological findings suggest that the enamel defect in mice is cuased by premature degeneration of ameloblasts.

3. Two alleles of the gene tabby in the mouse have different degrees of effect.

4. Two of the neural crest mutants (s^1 and ls) appear to have normal teeth.

5. Analysis of breeding data on fused and supernumerary molars in the rice rat suggests that the molar abnormality is under two types of genetic control. Major genes control the presence or absence of the abnormal complex but the degree of expression is under multigenic influence.

   Histological analysis of the developing dentition in the rice rat indicates that the abnormality may be of a type similar to that described by Dr. Sofaer in the tabby mouse.

6. Genetic studies of the traits of deafness, blue iris, lack of tapetum, and white fur in the cat have shown that the deafness can result from several origins. Congenital perceptive deafness may be due to the effect of a single dominant major gene which is also responsible for a total white coat. Another major gene at a different locus, which produces fur pigment spotting of a high degree, can also cause deafness. The traits of blue iris and lack of tapetum are highly but not perfectly correlated with each other and are both correlated with deafness.

7. Histological studies show that the bony form of the inner ear and the entire middle ear develop normally in the deaf white cats. The defect is of a cochleo-sacular type which can be detected first after birth as a failure of the organ of corti to develop although Reissner's membrane appears normal and the scala media is developed. A characteristic balling up of the tectorial membrane is also observed in profoundly deaf kittens. Reissner's membrane appears to collapse onto the Basilar membrane at a later time and the collapse may be incomplete.

   Histological and ophthalmoscopic examination of the eye in the white cats usually shows a lack of the tapetum in the eyes with a blue iris. Exeptions with tapetum and blue iris and no tapetum and pigmented iris have also been observed. The lack of tapetum affects the response to light as measured by pupil diameter of blue compared with brown eyes.

8. Examination of the reproductive performance and survival of white blue-eyed cats shows that many are sterile and that they have a higher death rate from disease than do pigmented cats. This propensity to disease is also shown in histological study of the ears of white cats in which purulent otitis media

is more common that in pigmented cats. Audiological study of the cats shows that some of the hearing defect in cats is of an intermediate, conductive type and is associated with histological purulent otitis media.

No abnormality of immunoglobulin or a complement could be demonstrated in white cats. Mitotic activity of lymphocytes in response to phytohemagglutinin was the same in white cats as in pigmented litter mates.

Since the neural crest has been reported to be the origin of odontoblasts an abnormality of neural crest might result in abnormality of dental development. Examination of the dental morphology and caries of white and pigmented cats in our colony has not demonstrated any differences between them.

Significance to Dental Research:

The genetic and developmental analysis of specific abnormalities in experimental animals can lead to a better understanding of related defects in man.

The establishment of appropriate analytical procedures for human data will provide a sound basis for future work in this field.

Neural crest cells are a part of the early ectoderm that gives rise to pigmentation and to the sensory neurons. 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 demonstration that two different major genes can produce similar disturbances of pigment and hearing the cats may have implications regarding the genetics of the Waardenburg's syndrome which has been thought to be due to one major gene.

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 not included.

Serial No. NIDR-21 (66)
1. Human Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Teratogenic Effects in the Fetus and Metabolic Changes After Hypervitaminosis D in the Pregnant Rabbit

Previous Serial Number: NIDR-52

Principal Investigators: Dr. M. V. Barrow
Dr. L. F. Mills, Biometry and Field Investigations Branch

Other Investigators: Dr. D. R. Bergsma

Cooperating Units: W. F. Friedman, Chief of Pediatric Cardiology, University of California, San Diego, California

Man Years

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

Project Description:

Objectives:

Hypervitaminosis D in pregnant rabbits has been reported to produce anomalies of the orofacial complex, as well as enamel hypoplasia and cardiovascular abnormalities. The objectives during the current year were to:

a. study the effects of hypervitaminosis D on the fetus in terms of overall growth rate and development throughout gestation.

b. measure calcium and phosphorous levels in dams and fetuses throughout gestation, and during the neonatal period; and to compare these data with those obtained in control animals. This was done in an attempt to identify derangements in calcium and phosphorous metabolism and to correlate such changes with anatomical defects.

Methods Employed:

Pregnant rabbits were treated with 100,000 units of vitamin D_2 three times weekly beginning on the eighth day of gestation until a total of 800,000 units had been given (by day 24). Maternal blood samples were drawn beginning on day 5 and on day 8 and 15. Animals were killed and litters taken on day 20, 24, 27, 30, 31, and 32, and fetal blood as well as maternal blood were collected. Maternal and fetal samples were also obtained one week, two weeks, one month and three months postpartum. Blood was analyzed for calcium and phosphorus and the fetuses were either cleared or grossly dissected after fixation.

Major Findings:

Severe growth retardation was noted in fetus from treated mothers. In addition the fetuses also showed a significant incidence of ocular defects (see NIDR-22) and anterior crossbite, and virtually all had hypodontia and enamel hypoplasia. In control pregnant animals both calcium and phosphorus levels decreased near term. In treated animals, the calcium levels did not decrease, while the phosphorus levels were significantly elevated above normal. Levels of calcium and phosphorus were not appreciably different in treated and control fetuses.

Significance to Dental Research:

The experimental findings in the rabbit fetuses resemble somewhat the enamel hypoplasia and malocclusion seen in the human in the syndrome of idiopathic hypercalcemia of infancy or supravalvular stenosis syndrome. As such, they may be an acceptable model for intensive histopathological and metabolic studies. In addition, 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:

Histological sections of teeth, bone joints and cardiovascular system during specific periods of gestation are in progress to correlate with gross anatomical changes and metabolic derangement. Protuberant eyes and glaucoma are to be studied by Dr. Donald R. Bergsma (see Project No. NIDR-22).

Part B

Publications:

1. Friedman, W. F. and Mills. L. F.: The relationship between vitamin D and the craniofacial and dental anomalies of the supravalvular aortic stenosis syndrome. Pediatrics 43: 12-18, 1969.

Serial No. NIDR-22 (66)
1. Human Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

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: NIDR-35

Principal Investigator: Dr. D. R. Bergsma

Other Investigators: Dr. M. V. Barrow
Dr. L. F. Mills, Biometry and Field Investigations Branch

Cooperating Units: W. F. Friedman, Chief, Pediatric Cardiology, University of California, San Diego, California

Man Years

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

Project Description:

Objectives:

1. To differentiate the occurrence of spontaneous (genetic) buphthalmos in rabbits from that caused by exposure to vitamin D in utero, and further to investigate the possibility that vitamin D may act to facilitate expression of the genetically-determined predisposition to buphthalmos (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 described in NIDR-21.

Methods Employed:

Originally, one normal and one buphthalmic male were obtained as stud sires, and 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. Initial high offspring mortality delayed the project until the major factors responsible were identified, and, where possible, corrected. Three adverse factors could not be changed, namely: (1) seasonal reproductive and survival variation, (2) decreased fecundity in the buphthalmic line, and (3) poor offspring survival after exposure to vitamin D_2 *in utero*. Therefore, a second normal and a second buphthalmic stud male and two more normal female breeders were obtained to partially compensate for offspring mortality. Moreover, the colony was allowed to proliferate for one generation before vitamin D_2 was given to pregnant females, since this regimen is usually incompatible with the production of additional litters. Thus, selected females have been allowed to produce one or two control litters before being given the vitamin.

During this stage of the experiment, intraocular pressure measurements have been obtained on animals of each genotype, and selected histological studies are being obtained. The incidence of buphthalmos in the vitamin D_2 exposed group will be compared with appropriate controls for each genotype.

Major Findings:

Intraocular pressure measurements to date have been compatible with reports in the literature that buphthalmos is inherited as an irregularly expressed recessive trait in the NIH rabbits. The overall high mortality among vitamin D_2 exposed offspring experienced by Dr. L. F. Mills on a small pilot group in 1967 has been confirmed in this study. Because of high mortality, insufficient data is available at present to establish comparisons between the effects of vitamin D_2 on the genetically distinct types of rabbits being tested.

Significance to Dental Research:

The relationship between exposure to vitamin D and genetic background in producing the abnormalities described in this project and in project NIDR-21 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, while the causes of buphthalmos in humans are undefined, there is evidence that both this and other types of glaucoma have a genetic component.

Proposed Course of Project:

The numbers of rabbits being bred have been increased to partially offset high mortality. The dosage experiments are now being performed sequentially instead of concomitantly in order to reduce the number of variables operating on any group of animals. Increased reliance is being placed on control comparisons with treated and untreated genetically normal animals in the parent project. Finally both increased use of histology and earlier methods of diagnosis of buphthalmos in living offspring are being evaluated to offset offspring mortality.

Part B not included.

Serial No. NIDR-23 (62)
1. Human Genetics
2. Developmental Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

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-29

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 Stroke, NIH and School of Public Health, University of Hawaii

Man Years

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

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:

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). The resulting coded data is being processed at the NIH Computer Center, utilizing the SEGRAN program, developed by Dr. Chung.

Approximately 300 deaf individuals who are present or former pupils at the Clarke School and 700 of their unaffected relatives including siblings, parents and grandparents have been examined for variations of the eye, ear, mouth, teeth, face, neck, pigmentation of head and neck and audiometric examination. Among the deaf 170 young people also had caloric and turning chair tests of vestibular function and an electrocardiogram. All but the youngest children were tested for ability to taste PTC, and had a urine sample tested for glucose protein, phenyketones and similar compounds and a microscopic examination for cells.

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 NINDS support to carry out the serum studies, exclusive of the electrophoresis.

Patient Material:

Patient material consists of the students at the Clarke School for the Deaf, their siblings, parents and other relatives, if indicated. Alumni of the Clarke School and their families were examined, including as many of the graduates since 1930 as are still available. This sample comprises approximately 1,500 people, 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 percent of pupils attending Clarke School since 1930, 96 percent of those attending since 1920 and 47 percent 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 percent of the childhood deaf were the result of classifiable disease, accident or toxic condition. An additional 4 percent resulted from unclassifiable, but probably extrinsic causes, while 76 percent were the result of hereditary factors or undetected

disease. Genetic analysis further suggests that undetected disease accounts for 18 percent of the total, so that 58 percent of the defects observed have a simple hereditary basis.

About one-third of the cases of deafness due to extrinsic agents are the result of measles or other acute central nervous system diseases. Severe prematurity, which may be confounded with the affects of maternal rubella, accounts for about one-fourth of the extrinsic cases. Clearly defined, prenatal rubella is present in 14 percent. The remainder is due to a variety of causes, each less than 5 percent of the total.

Genetic analysis shows that simple recessive mendelian factors account for about 40 percent 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 percent 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 in 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.

Genetic information about the way congenital defects are distributed in families and populations is basic to understanding of human malformation. The identification and characterization of a particular type of defect involving abnormalities of hearing, eye, facial morphology, and pigmentation in man and an analog or homolog in the domestic cat provides a possible experimental animal system for further study of a frequent type of human deafness. Interpretation of the observations in light of embryological information about the action of the neural crest suggests a rational basis for experimental study.

Proposed Course of Project:

The data collection phase of the study of the Clarke School pupils and relatives has been completed. The statistical and genetic studies on the data are in progress, and this work will continue for at least one additional year. Analysis of 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 not included.

Serial No. NIDR-24 (54)
1. Human Genetics
2. Developmental Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Environment and Genetic Factors in Taste and Smell Abilities

Previous Serial Number: NIDR-30

Principal Investigator: Dr. K. S. Brown

Other Investigators: Mr. C. J. MacLean and Mr. W. C. Leyshon

Cooperating Units: None

Man Years

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

Project Description:

Objectives:

1. To describe individual variation in the ability to detect the odors and tastes 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 gustatory and olfactory thresholds 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. Two thousand school children for a study of six odors and the ability to taste phenylthiocarbamide (PTC).

2. Eighty pairs of parents and their children in a study of two odors and ability to taste PTC.

3. One hundred and fifty students in a study of the correlation between ability to taste PTC and the ability to taste anetholetrithione.

4. A series of twins are being examined for threshold to isobutyric and isovaleric acids which have been suggested as showing large individual differences in threshold.

Major Findings:

The threshold for the ability to smell chemical compounds is controlled by several factors. A general sensitivity factor affecting threshold to all odors is the major influence. Special factors appear to affect the threshold for particular classes of chemicals. Preliminary evidence has identified two of these factors, degree of ionization as an acid and degree of ionization 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.

Significance to Dental Research:

Taste and smell are the least known of the special senses. The mechanisms involved are unknown and only a few studies of the genetic aspects of these abilities have been attempted. Application of genetic and statistical techniques of analysis to quantitative threshold data on perception of specific chemical compounds may

increase understanding of the mechanisms involved.

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:

1. Brown, K. S., MacLean, C. J. and Robinette, R. R.: The distribution of the sensitivity to chemical odors in man, Human Biology 40 (4): 456-472, 1968.

Serial No. NIDR-25 (62)
1. Human Genetics
2. Developmental Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Saliva Study

Previous Serial Number: NIDR-36

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 new and various techniques as they are developed to determine, define and study the 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 study the etiology of the deviations from the normal variations with emphasis on oral conditions.

4. To study salivary component change in selected disease entities.

5. To relate salivary components to genetic control.

6. Determine if human salivary isoamylases are under genetic and/or physiological control.

7. Study possible genetic/environmental control of lysozyme in saliva and other body fluids.

8. Further investigation of factors in parotid saliva which correlate positively with the rate of calculus formation.

9. 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. Lysozyme isoenzyme detection in saliva, serum and urine after disc electroporetic separation.

3. 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).

5. 200-300 whole saliva samples from NINDS twin study to determine salivary isoamylase heritability coefficients.

6. Patients requiring radiation for various diseased states who subsequently have the pancreas or salivary glands radiated.

Major Findings:

An ultrafilterable biuret positive material was reported found in human parotid saliva which was positively correlated with the rate of dental calculus formation.

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.

Familial patterns have been demonstrated in the isoamylase patterns of saliva but genetics are not certain.

Irradiation to salivary glands does not appreciably change the salivary isoamylase pattern within the radiation levels so far studied. In corroboration with other researchers' findings it does alter the pattern in the blood serum and urine.

Significance to Dental Research:

When the genetics of the salivary isoamylases is understood it will provide another or other genetic loci, for mapping man's chromosomes, with focus on the oral cavity.

It is possible that the compound correlated with calculus if further studied will provide greater insight into calculus etiology and control.

The contribution of the salivary glands to elevated serum amylase in the diagnosis of pancreatitis can now be estimated and should help eliminate those high serum amylases due to salivary gland disorders.

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.

5. Investigate the physical differences of the isoamylases.

Part B

Publications:

1. Wolf, R. O.; Taylor, L. L.; Croft, L. K.; and MacLean, C. J.: Parotid saliva analysis in relation to dental calculus formation rates. J. Periodontology, 40 (2): 5-11, 1969.

Serial No. NIDR-26 (67)
1. Human Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Studies on Site of Action of Phytohemagglutinin on Circulating Human Lymphocytes

Previous Serial Number: NIDR-33

Principal Investigator: Dr. J. W. Graef

Other Investigators: Dr. H. L. Cooper, Laboratory of Biochemistry
Dr. S. Handmaker, Laboratory of Biochemistry

Cooperating Units: None

Man Years

Total:	1
Professional:	1
Other:	0

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.

In addition, nuclei are prepared from cells incubated with phytohemagglutinin for varying lengths of time up to 72 hours. RNA polymerase is assayed for 5 minutes to 2 hours to determine kinetics of RNA polymerase system under varying assay conditions in stimulated and unstimulated cells.

Patient Material:

Normal volunteers.

Major Findings:

Even after 3 hours of incubation with phytohemagglutinin, nuclei are not directly affected so far as can be measured by uptake of nucleotide precursor. However, it is possible to enhance activity of unstimulated nuclei by the addition of cytoplasm from stimulated cells.

In addition, the activity of the phytohemagglutinin stimulated RNA polymerase system in isolated nuclei is greater than unstimulated under all assay conditions, although the system appears to degenerate more rapidly when assayed in solutions of high ionic strength.

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 function 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:

Having demonstrated the capacity to stimulate inactive nuclei by reconstituting cells, it is proposed to determine concentrations and conditions for this effect. In addition, efforts to examine more quantitatively increased activity of RNA polymerase enzyme using kinetics experiments with a view toward examining the affinity constants for the enzyme with and without exposure to phytohemagglutinin are being pursued.

Part B not included.

Serial No. NIDR-27 (63)
1. Human Genetics
2. Population Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Genetic Studies of Oral Diseases, Anomalies and Development

Previous Serial Number: NIDR-31

Principal Investigator: Dr. D. W. Runck
Dr. J. D. Niswander

Other Investigators: Dr. C. S. Chung, University of Hawaii
Dr. P. L. Workman

Cooperating Units: School of Public Health, University of Hawaii

Man Years

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

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 three areas: (1) survey of oral conditions of high school children in Hawaii, (2) survey of school children in Hawaii who have had orthodontic treatment and (3) survey of dental cephalometric X-rays of approximately 1,000 Hawaiian school children to investigate the frequency of congenitally missing teeth. 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. During the past year data collection was completed.

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 groups 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 percent 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.

Other sources of data for individual studies are also utilized. One example is the data collected in Japan as part of the inbreeding studies as well as data collected on the Papago Indians. These data are still quite useful for a number of specific smaller projects.

Major Findings:

Examinations are now completed for 17,772 children of intermediate and high school ages covering 16 schools on the island of Oahu and 2 schools on the island of Hawaii. Each individual has information on dental caries, P.I., O.H.I.S., 10 items of occlusion characteristics, oral clefts, tori, 5 items of cephalic measurements, weight, and height in addition to I.D. information and history of orthodontic treatment. Further, about 4 percent of the sample have surface caries evaluation on a selected number of teeth and about 300 students have partial dental impressions.

These data have been linked by computer with the birth certificate information on those individuals born in Hawaii. The birth certificate information includes race of the parents, occupation of father, birth order, birth weight, maternal age, abnormal labor, maternal year during pregnancy, birth injury, etc.

Analyses of all physical and dental characteristics have been performed first for study of the possible effects of epidemiological factors, and second for study of racial factors on the statistical model of diallele cross. The results may be briefly summarized as follows:

1. The highest rate of dental caries was observed among the children of Japanese and Hawaiian ancestry.

2. P.I. and O.H.I.S. are in general lower for Oriental children than Caucasians, but Hawaiians showed the highest scores in both respects.

3. Hawaiians tend to have earlier eruption of third molars than other racial groups.

4. Orientals and Hawaiians have higher cephalic index than Caucasians.

5. All cephalic measurements are subject to considerable amount of environmental variation.

6. Hawaiians exhibit higher degree of obesity even at this age range.

7. Birth weights show significant positive correlations with practically all cephalic and physical measurements.

8. Children of Hawaiian ancestry show best occlusion among all races.

9. Orientals and Hawaiians in general have more Type III (angle) malocclusion than Caucasians, whereas Caucasians have more Type II malocclusion.

10. Orientals have higher frequency of relatively minor malalignment than other racial groups.

11. There were no detectable effects of the recombination or hybridity in the dental traits studied.

12. Body weight shows a degree of dominance in the major racial crosses.

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 proceed with analysis of the data.

Part B

Publications:

1. Bailit, H. L.; Niswander, J. D.; and MacLean, C. J.: The relationship among several prenatal factors and variation in the permanent dentition in Japanese children. Growth 32: 331-345, 1968.

2. Bailit, H. L.; Workman, P. L.; Niswander, J. D.; and MacLean, C. J.: Dental asymmetry as an indicator of genetic and environmental stress in human populations. Amer. J. Phys. Anthrop. (in press).

Serial No. NIDR-28 (67)
1. Human Genetics
2. Population Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Theoretical and Applied Analyses in Human Populations with Particular Emphasis on the Study of Genetic Variation.

Previous Serial Number: NIDR-32

Principal Investigator: Dr. P. L. Workman

Other Investigators: Mr. C. J. MacLean, Mr. W. C. Leyshon, Dr. J. D. Niswander, Mrs. B. Y. Iba and Miss R. Singleton

Cooperating Units: None

Man Years

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

Project Description:

Objectives:

1. 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.

2. To develop methods for determining which 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 local differentiation.

3. To obtain indicators of developmental instability in humans which can be used to compare populations under genetic stress (severe inbreeding for example) or environmental stress (poor nutrition, high disease rates). In particular we have studied

developmental asymmetry with respect to measurement characters of homologous teeth.

Methods Employed:

Methodology in this area involves both data analysis and more theoretical mathematical procedures, ranging from model building to computer simulation. The major sources of data are: (1) the genetic information obtained from field studies of the Papago and Pima Indians in conjunction with the social, demographic and genealogic information available in the tribal registers, and (2) genetic data obtained from field studies on the Zuni Indians.

Major Findings:

1. There is significant local genetic differentiation in the Papago, in large part related to partial geographic isolation among tribal subunits. The intra-tribal differences are considerably greater then that observed between Papago and Pima mean tribal frequencies. The genetic relations among Southwest Indian tribes closely parallel the linguistic affinities among these groups.

2. In most populations, whether large and panmictic or small and affected by differential fertility, genotype frequencies are generally not different from those predicted by the Hardy-Weinberg Law. The forces usually affecting genotypic frequencies (population sizes, selection, subdivision, etc.) have been shown to have opposite (and counterbalancing) effects on the genotypic distribution. That is, the so-called fixation index, F, which describes the net effect of all forces acting on the genetic structure is usually close to zero. The variation in estimates of F from both theoretical and real populations of different size and structure has also been described.

3. Comparisons among four populations subject to varying amounts of environmental stress has shown that dental asymmetry provides a useful indicator of such differences in stress.

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:

1. Allard, R.; Jain, S. K.; and Workman, P. L.: The genetics of inbreeding populations. Advances in Genetics 14, 1968.

2. Workman, P. L.: F-Statistics and the description of polymorphic loci. Am. J. Hum. Genet. 21(1): 99-100, 1969.

3. Workman, P. L.: The analysis of simple genetic polymorphisms. Human Biology (in press), 1969.

Serial No. NIDR-29 (58)
1. Human Genetics
2. Population Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Genetic Studies of Oral Clefts and Other Major Congenital Malformations

Previous Serial Number: NIDR-37

Principal Investigator: Dr. J. D. Niswander

Other Investigators: Dr. M. V. Barrow, Mrs. B. Y. Iba, and Miss R. Singleton

Cooperating Units: PHS Division of Indian Health

Man Years

Total:	3 1/2
Professional:	1
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.

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. Our studies among the families of cleft children from Pennsylvania have not confirmed a difference in facial morphology among the "unaffected relatives" reported by other workers.

2. Analysis of the Michigan and Montana data shows no significant temporal clustering of oral cleft births. Analysis was designed to detect clustering by season within years as well as cycles as long as 5-7 years.

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. Micropthalmia 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. More detailed follow-up of the Papago tribe revealed 7 cases of myelodysplasia. Incidence was estimated at approximately 1 case/200 births. All cases clustered in one of two extended family groups.

4. Oral clefts occur with a frequency of approximately 1:400 births in the American Indian. Cleft lip $\pm$ cleft palate is 2-3 times more frequent than isolated cleft palate, however, there is considerable variation among subgroups in this regard. Among the Athabascans 30 clefts involving the lip occurred but only 2 cases of isolated cleft palate were found. No relationship has been detected between the frequency of clefts and the amount of Caucasian admixture.

5. Sex-ratio for births from marriages between tribes is significantly reduced compared to that for marriages within a particular tribe. The meaning of these results is unclear. They are compatible with several biological as well as social interpretations.

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 for approximately one more year. 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 not included.

Serial No. NIDR-30 (69)
1. Human Genetics
2. Population Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Mathematical and Numerical Methods in Genetics Analysis

Previous Serial Number: None

Principal Investigator: Mr. C. J. MacLean

Other Investigators: Dr. P. L. Workman

Cooperating Units: None

Man Years

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

Project Description:

Objectives: Subproject A--Population Searching Program

1. To produce a computer program package for use in genetic analysis of pedigree data with regard to:

   a. inbreeding coefficients
   b. coefficients of relationship
   c. graphical pedigree displays
   d. clone tracing of founders
   e. disclosure of all relatives of specific individuals.

2. To carry out the calculations on population data tabulated by other investigators in the Human Genetics Branch.

3. To prepare the program and documentation for dissemination.

Methods Employed:

The program system was designed and produced in the Human Genetics Branch. It was tested and debugged on the time-sharing, teletypewriter-computer.

Major Findings:

The population searching program has been used in several genetic analyses in the Human Genetics Branch and has proved to be a rapid efficient calculator of pedigree information.

Significance to Dental Research:

Programming techniques were developed, including list processing, binary coding of pedigree structure and efficient storages of population data, which are appliable to genetics programs to be produced in the future.

Proposed Course of Project:

The program development is finished. The program is available for use by any investigator in NIDR or outside.

Objectives: Subproject B--Estimation and Statistical Testing of Birth and Death Rates

1. To investigate the relationship between birth and death rates which vary over time with respect to the resulting population size and age structure.

2. To develop estimation and statistical testing techniques by which to analyze birth and death rates from population data.

3. To develop techniques for applying variable rate analysis to the many other problems in population genetics in which such phenomena occur.

Methods Employed:

1. Mathematical analysis of theoretical population models is being conducted in which the usual assumption of constant birth rate λ is replaced by a realistically varying rate, $\lambda(t)$, over time t. This investigation consisted in establishing the differential equations to represent the population growth and solving them. The analysis is supported by numerical experiments carried out on a computing machine.

2. Estimators of $\lambda(t)$ are devised and analyzed by numerical experiment. Hypothesis testing procedures are established, using the estimates above, to test the general form of $\lambda(t)$.

Major Findings:

The result in population structure of variable birth and death rates is very complex, but analytical work can be conducted by use of an integral transformation of the time scale. Transforming all the variables into this special time scale reestablished between them the independence, or orthogonality, which made the original constant λ model tractable.

Maximum likelihood estimators and method of moments estimators, keep their qualities with respect to consistency, efficiency, bias, etc., in the transformed time scale, but least squares estimation is rendered useless.

Hypothesis tests can be designed specially to test the qualities of independence and identical distribution, which are induced by the correct transform but obviously not by a false one, to test the validity of the hypothesized transform.

Significance to Dental Research:

Better understanding of population structure with realistic birth and death rates would help geneticists in many phases of investigation.

Proposed Course of Project:

After statistical techniques are found to be efficient by the simulation experiments being conducted on a computer, they must be applied to real population data; first bacterial cultures, finally human population. There is a great deal of data available in the literature which requires analysis by the methods being developed.

Part B

Publications:

1. MacLean, C. J.: Alternative programming techniques for inbreeding calculations. Proceedings of International Conference on Computer Applications in Genetics 1968 (in press).

Report of the Biometry and Field Investigations Branch
National Institute of Dental Research
Summary Statement

Major activities of the Biometry and Field Investigations Branch involved the clinical testing of the anticaries effect of repeated topical sodium fluoride applications in mouthpieces and methodologic research into the design and statistical analysis of dental clinical trials.

In addition to further information on the efficacy and duration of effect of fluorides applied frequently to children in custom-fitted mouthpieces, field studies completed and in progress have provided collateral data on fluoride uptake of treated teeth. These data illustrate the necessity and feasibility of establishing a therapeutic rationale for fluoride formulations to be tested against caries, as contrasted with the empiricism often characteristic of previous fluoride studies.

Substantial progress was made toward solution of the several statistical problems involved in design, estimation and tests of hypotheses in caries clinical trials.

The Chief, Clinical Trials Section and the Chief, Biometry Section served as members of an A.D.A. Task Force on the Design and Analysis of Dental Clinical Trials.

Chronic shortages of professional staff continued; as a consequence all field studies were undertaken on a collaborative basis. Data collection, processing, analysis, and interpretation involved in these studies were conducted and supervised by Branch personnel.

Dental Caries

Significant residual anticaries effects were found in children 23 months after they had received at least 200 repeated topical applications in mouthpieces of either a plain or acidulated NaF gel. Treated children had approximately 70% fewer new decayed teeth than untreated controls, a protection of the same order as that observed immediately after the completion of active treatment. Enamel fluoride concentrations remained high in exfoliated deciduous teeth collected 6-9 months after treatment. The data offered suggestive evidence that the maintenance of approximately 2000 ppm fluoride in the outer 5-10 microns of enamel may be desirable for optimal anticaries effect.

Thrice-weekly applications in mouthpieces of a NaF gel in children who had consumed optimally fluoridated water since birth showed small, non-significant, anticaries effects after 18 months. In this study, untreated control children, consuming fluoridated water from birth, had an extremely low incidence of caries which mitigated against demonstrating a possible additive anticaries effect of the topical fluoride regimen. This study will continue until the end of May 1969 when the results of 30 months of treatment will be evaluated.

A study of the potential of repeated topical applications of sodium fluoride gels in the prevention of carious lesions in the caries-free deciduous dentition continued. This investigation will be concluded in June 1970 and involves dependents, aged 2-7 years, of U.S. Coast Guard personnel.

Transmissibility of certain forms of caries-conducive streptococcal strains (S. mutans) in laboratory animals has been repeatedly demonstrated. A study has been initiated to study the mode of transmission of S. mutans within family groups. S. mutans has been isolated from several mothers of young children who do not harbor these microorganisms. These streptococci have been labelled by making them resistant to streptomycin, and attempts have been made to reimplant them into the mouths harboring the parent strain. These families are being closely monitored to detect possible pathways of transmission of the labelled pathogens.

Several laboratory studies were completed during the year, as adjuncts to planning for further clinical trials. One pertinent finding was that several chemical forms of dietary phosphate additives had little effect against cavitation in the hamster, when fed intermittently as has been attempted in some human studies. Previously documented anticaries effect of phosphates when given in all food consumed was reconfirmed, but animals on the most effective regimen (tri-meta phosphate) showed some evidence of malaise.

Biometric and Collateral Activities

The completion of a data processing system, using high speed computers for summary and analysis of the results of clinical trials, has effected a drastic reduction in the time formerly required for such analyses. In addition, it permits more refined breakdowns of the collected caries incidence data. For example, the caries experience in pitted and smooth tooth surfaces and in anterior and posterior teeth is now being routinely assessed separately. This project has demonstrated the feasibility and economy of a fully automated system which may be used without modification by many clinical investigators and which facilitates direct comparisons among the results of different studies.

Computer programs were also written for a number of other research applications, including the summary and analysis of dental caries in laboratory animals and an evaluation of the reliability of epidemiologic indices of malocclusion.

The continuing program of research into the methodology of design and analysis in dental clinical trials resulted in several important findings during the year. These included the demonstration that an assumption critical to previous mathematical models of the dental trial is untenable. This led to development of an improved and simplified method of unbiased estimation of caries attack rates. As the variance of this estimate was also derived, this research had immediate practical application in the testing of hypotheses in clinical trials.

Professional personnel of the Biometry Section engaged in a variety of consultant activities in statistics and computing for Institute scientists as well as for dental researchers in several universities, the Army, the Veteran's Administration, the National Health Survey, the Food and Drug Administration and State Health Departments.

Malocclusion

An adequate method for assessment of malocclusions in populations has been badly needed. A recently proposed composite index, the Treatment Priority Index, appears to have promise for this purpose.

Preliminary results of a study of the TPI suggest that inter- and intra-examiner reproducibility of scores, derived from measurement of dental casts, are satisfactory, and that examiners require only brief training to use the index properly. Thus far, no meaningful method of assessing the validity of the TPI has been found.

A study has been initiated in an attempt to develop a new descriptive classification of malocclusion, based upon 18 morphologic characteristics, and using technics of numerical taxonomy or "cluster analysis." If successful, development of an epidemiologic index with higher information content than those now in use may be possible.

Training Activities

The Branch was fortunate in being able to support a dental officer as a full-time trainee in biostatistics and field investigations. This individual was introduced to a variety of field and laboratory research technics and experiences, and to the principles of data processing and analysis. He will be assigned to the University of California, School of Public Health in July, 1969, for formal study leading toward the M.P.H. degree in epidemiology.

In addition, two candidates for the Doctor of Public Health in epidemiology were assigned for short-term summer field training.

The Director, Clinical Trials Section, gave lectures in cariology and field research to dental students at Howard University, the University of Maryland, the U.S. Naval Dental School, the U.S. Army, and to various universities and dental societies.

Serial No. NIDR-31 (64)
1. Biometry and Field Investigations Br.
2. Clinical Trials Sec.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Clinical Anti-Caries Effect of Repeated Topical Sodium Fluoride Applications by Mouthpieces

Previous Serial Number: NIDR-56

Principal Investigator: Dr. H. R. Englander

Other Investigators: Dr. J. P. Carlos

Cooperating Units: Cheektowaga Central School District No. 1; James R. Mellberg, Research Division, The Kendall Company, Barrington, Illinois

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description

Objectives:

The original purpose of this project was to test the anticaries effect of water soluble gels containing 1.1 per cent NaF 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 regimen of repeated topical NaF applications was discontinued in June 1966. The principal purpose of the present study was to assess their residual anticaries effect 23 months later (May 1968).

Methods Employed:

Clinical examination of 379 children, initially aged 11-14 years, who had been randomly assigned to groups receiving the sodium fluoride gels and a control group not receiving the gels.

Part A (continued)

Statistical evaluation of data collected during the 21-month period in which the gels were applied, and of data collected 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:

The data from this study show that 379 children, originally aged 11-14 years, who had received at least 200 daily topical treatments with either an acidulated NaF phosphate or plain NaF gel over a period of 21 months, continued to have significantly lower dental caries increments 23 months after the treatments had been discontinued than were found in an untreated group.

An analysis of the fluoride concentration of exfoliated deciduous teeth collected during the posttreatment period showed high residual fluoride concentrations remaining in the four outer layers of enamel.

Neither the clinical caries data nor the enamel fluoride concentration of the deciduous teeth showed evidence of a reduction in the magnitude of the protective effect during the 23-month posttreatment period.

Significance to Dental Research:

This project has demonstrated the striking residual anticaries benefits that can be achieved with repeated topical NaF treatments by mouth applicators in children drinking fluoride-deficient water. Furthermore, it has demonstrated the importance of considering fluoride uptake of teeth in evaluation of clinical trials with fluorides.

Proposed Course of Project:

This project has been completed.

Part B

Publications:

1. Englander, H. R., Keyes, P. H., and Gestwicki, M.: Clinical anti-caries effect of repeated topical sodium fluoride applications by mouthpieces. JADA 75: 638, 1967.

Part B (continued)

2. Mellberg, J. R., Englander, H. R., and Nicholson, C. R.: Acquisition of fluoride in vivo by deciduous enamel from daily topical fluoride applications. J. Oral Ther. & Pharm. 3: 330, 1967.

3. Mellberg, J. R., Englander, H. R., and Nicholson, C. R.: Acquisition of fluoride in vivo by deciduous enamel from topical fluoride applications over 21 months. Arch. Oral Biol. 12: 1139, 1967.

4. Englander, H. R.: Views on the rationale of topical fluoride therapy. JACD 35: 15, 1968.

5. Englander, Harold R., Carlos, James P., Senning, Rickley S., and Mellberg, James R.: Residual anticaries effect of repeated topical sodium fluoride applications by mouthpieces. JADA 78: 783-787, 1969.

Serial No. NIDR-32 (68)
1. Biometry and Field Investigations Br.
2. Clinical Trials
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Anti-Caries Effect of Repeated Topical Sodium Fluoride Treatments on the Deciduous Dentition

Previous Serial No.: NIDR-58

Principal Investigator: Dr. H. R. Englander

Other Investigators: Dr. H. V. Jordan and Dr. J. P. Carlos

Cooperating Units: United States Coast Guard, Governors Island, New York, N. Y., Captain William O. Engler and Commander Edward D. Woolridge, U.S.P.H.S.
Amount FY '69 - $27,550
Dates 6/1/67 - 6/30/70

Man Years:

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

Project Description

Objectives:

The principal purpose of this project has been to determine whether daily topical applications of a concentrated sodium fluoride gel can maintain the initially caries-free deciduous dentition of preschool 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

Part A (continued)

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.

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.

Introducing labelled strains of native S. mutans into the mouths of mothers to follow transmission within the family.

Methods Employed:

Clinical and radiographic examinations of approximately 300 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 from a sample of the children and culturing for cariogenic streptococci.

Major Findings:

Clinical dental examinations conducted six months after the applications had been initiated showed that caries increments in the control group were too small to evaluate the effect of the treatments so soon.

Caries-free 2-year old children do not appear to harbor cariogenic streptococci. Labelled strains of S. mutans have been reimplanted in 20 mothers. In two cases, these strains have persisted in the mouth for two months.

Adults with full upper and lower dentures can harbor S. mutans.

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

Part A (continued)

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 may provide information concerning the optimum fluoride concentration necessary for a maximum anti-caries effect. Studies on the plaque microflora will provide insight into the relations between the cariogenic streptococci and smooth surface, and pit and fissure decay.

The implantation studies will make it possible to demonstrate the role of infection in dental caries, and provide insight into the mode of transmission of S. mutans within the family.

Proposed Course of Project:

The repeated topical fluoride applications will continue for another year. Clinical examinations have been conducted in May 1969, and this clinical caries data will be analyzed for any differences in dental caries increments. Labelled strains of S. mutans will continue to be reimplanted in additional mothers.

Part B Not included.

Serial No. NIDR-33 (66)
1. Biometry and Field Investigations
2. Clinical Trials
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Anti-Caries Effect of Repeated Topical Sodium Fluoride Applications in a Fluoridated Community

Previous Serial Number: NIDR-46

Principal Investigator: Dr. H. R. Englander

Other Investigators: Dr. J. P. Carlos and Dr. H. V. Jordan

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

Project Description

Objectives:

This study is being supported by contract number Ph 43-67-60. The principal purpose of the project has been 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 were intended to be made three times weekly for three minutes over a period of three years.

Exfoliated deciduous teeth and extracted permanent bicuspids removed for orthodontic purposes have also been collected from the children in order to determine how much additional fluoride can be acquired by enamel from the topical treatments compared with that acquired from only consuming fluoridated water.

Part A (continued)

The occurrence and distribution of caries-conducive streptococcal strains in the dental plaque of children in Charlotte have been determined as part of an epidemiologic survey. The effect of the fluoride gel applications on the prevalence of this strain in the plaque has been 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. Sampling dental plaque for the presence of cariogenic streptococci.

Major Findings:

After approximately 18 months, dental caries increments in both experimental and control groups were very low; therefore it was too early to draw conclusions regarding the efficacy against caries of repeated topical fluoride applications in the native Charlotteans.

The teeth treated with the NaF gel have progressively acquired more fluoride as the number of treatments have increased. For example, the outer 5-10 microns of enamel of deciduous and permanent teeth receiving about 200 topical treatments have over 2,000 ppm fluoride as compared with 900 ppm fluoride for untreated teeth from children consuming only fluoridated water.

The repeated fluoride applications did not alter the occurrence and distribution of cariogenic streptococci.

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 and the microbiological phases of this study have provided valuable information on the pattern of fluoride uptake in deciduous and permanent teeth and the effect of repeated topical fluoride applications on streptococci with a cariogenic potential.

Proposed Course of Project:

Clinical dental examinations have been conducted on all children in May 1969, and the data is being processed in order to determine whether a difference in caries increment exists after 30 months.

Part B

Publications:

1. Mellberg, James R., Nicholson, Clyde R., Miller, Barry G., and Englander, Harold R.: Acquisition of fluoride *in vivo* by enamel from repeated topical sodium fluoride applications in a fluoridated area: a preliminary report. J. Dent. Res. 47 (No. 5), 733-736, 1968.

2. Jordan, H. V., Englander, H. R., and Lim, S.: Potentially cariogenic streptococci in selected population groups in the Western hemisphere. JADA 78: 1331, June 1969.

Serial No. NIDR-34 (63)
1. Biometry and Field Investigations Br.
2. Clinical Trials
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Experimental Dental Caries in the Syrian Hamster

Previous Serial Number: NIDR-48

Principal Investigator: Dr. H. R. Englander

Other Investigators: Dr. P. H. Keyes

Cooperating Units: None

Man Years

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

Project Description

Objectives:

To ascertain whether addition of phosphate compounds would affect plaque formation and the associated dental caries initiated on the smooth coronal surfaces of molars in the Syrian hamster. In other experiments the effect of vancomycin on S. mutans and dental caries was evaluated.

Methods Employed:

1. A caries-conducive ration was supplemented continuously or intermittently with either sodium trimetaphosphate, monosodium phosphate, diammonium phosphate, or sodium phytate.

2. A concentrated solution of vancomycin was applied topically to the teeth of hamsters.

Part A (continued)

Major Findings:

1. The findings from the experiments on the administration of phosphates indicate that a regimen of phosphate supplements tends to lose its retarding effect on cavitation unless administered continuously.

2. Repeated topical applications of vancomycin can eliminate S. mutans and inhibit carious lesions. S. mutans could not be recovered from the mouths of hamsters 6 weeks after the repeated topical applications of vancomycin had been discontinued.

Significance to Dental Research:

1. It is unlikely that phosphate compounds would have any important potential as an anticaries agent when consumed intermittently by human beings.

2. The potential of topical applications of vancomycin to eliminate S. mutans in man should be studied.

Proposed Course of Project:

Studies will continue to assess the antibacterial, antiplaque, and anticaries effect of vancomycin in the hamster.

Observations will be made of the length of time it takes for S. mutans to reappear in the mouths of hamsters after topical vancomycin applications have been discontinued.

Part B

Publications:

Englander, H. R., and Keyes, P. H.: Effect of phosphate supplements on cavitation in hamsters infected with caries-conducive streptococci. J. Dent. Res. (in press).

Serial No. NIDR-35 (68)
1. Biometry and Field Investigations Br.
2. Biometry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A:

Project Title: A Computer-Oriented Data Processing System for Dental Clinical Trials

Previous Serial Number: None

Principal Investigator: Dr. J. P. Carlos

Other Investigators: Mr. R. S. Senning, Miss J. A. Brunelle

Cooperating Units: None

Man Years

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

Project Description

Objectives:

1. To develop a complete system for the rapid and detailed recording, editing, summary and analysis of data gathered in dental caries clinical trials.

2. To describe and document the system in sufficient depth so that it can be offered as a "package" for use of investigators in other institutions.

Methods Employed:

The system is based upon a tooth-state code designed to be recorded on an optical mark page reader, from which magnetic tape records are automatically produced. A series of 3 computer programs, written by the investigators for use on the NIH 360 system, respectively create the desired file format, edit the data for inconsistencies or omissions and produce detailed tabulations and summary statistics as output. The number of treatment and control

Part A (continued)

groups that may be processed and the number of children per group is unrestricted.

Documentation, when complete, will be in the form of a fully detailed Procedural Manual describing the use of the entire system.

Major Findings:

Thus far the system has been used to process the results from 2 clinical trials. With 3 study groups and approximately 150 children per group, the elapsed time for complete processing is about 10 days including computer running time of 11 minutes. This is in contrast to the 3-5 months required using the former clerical processing system which produced considerably less detailed information.

Significance to Dental Research:

A more uniform and detailed approach to the presentation and analysis of data from dental clinical trials is badly needed. Methods previously used have generally been too varied and superficial to permit comparisons of results from different studies or detailed evaluation of the effect of the test agents. The system described offers a feasible solution to this problem.

Proposed Course of Study:

Documentation and minor modifications of the data system are proceeding and will be completed during the coming year.

Part B

Publications:

Carlos, J. P.: A computer-oriented data processing system for dental clinical trials. Proc. of the American Dental Association Conference on Clinical Testing of Cariostatic Agents. A.D.A., Chicago, Ill. In Press.

Serial No. NIDR-36 (68)
1. Biometry and Field Investigations Br.
2. Biometry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A:

Project Title: Studies on the Design and Analysis of Dental Clinical Trials

Previous Serial Number: NIDR-55 (68)

Principal Investigator: Dr. J. P. Carlos

Other Investigator: Mr. R. S. Senning

Cooperating Units: None

Man Years:

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

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:

A new mathematical model describing the interaction between diagnoses at sequential clinical examinations and the caries process was developed. The assumptions of this model were compared with those of other previous models. Data from several clinical trials were analyzed using both the traditional measures of caries

Part A (continued)

increment and the model caries attack rate. Results were compared. The sample variance of the model caries attack rate was derived.

The effect of various distributions of teeth about which there is uncertainty concerning a diagnosis of carious was investigated. Expected values for the proportions of these teeth in the different transition categories arising from sequential examinations were determined.

Multiple regression techniques were utilized to analyze the predictive value of a number of variables on subsequent caries incidence using several sets of clinical trial data.

Major Findings:

Previous models of dental clinical trials invoked strong, and perhaps unrealistic assumptions in order to estimate all their parameters. The new model requires only one, apparently realistic, assumption to yield a single estimate, the caries attack rate. Direction and magnitude of differences among the caries attack rates of various groups is consistent with those of traditional measures of increment.

If teeth about which the diagnoses are uncertain are distributed between the probabilities of zero and one of being classified as carious, the expected proportion of teeth in each of the four transitional categories will not be one quarter. The expected proportion of one quarter in each category was an assumption of previous models. Moreover, if the distribution is uniform through the interval, consistent diagnoses are twice as likely as inconsistent diagnoses.

Over the limited age range usually encountered in dental clinical trials, the only variable associated with increment is prior caries experience. However, this association is small and could be controlled by subsetting in the analysis. Random allocation is more desirable than balanced designs in dental clinical trials.

Significance to Dental Research:

The design and analysis of dental clinical trials have often proceeded in the past without adequate attention to principles of clinical experimentation. The investigations undertaken in this project should yield methods which are well grounded in theory and have the proper rationale for their utilization.

Part A (continued)

The overall intent is to provide a scientifically valid basis of knowledge for design and analysis in dental clinical investigation.

Proposed Course of Project:

Continue the project investigating the new model and its assumption. Further multiple regression studies of "predictor" variables are in progress.

Part B:

Publications:

Carlos, J. P. and Senning, R. A.: Error and bias in dental clinical trials. J. Dent. Res. 47:142-148; Jan-Feb, 1968.

Presentations:

Senning, R. S. and Carlos, J. P. An improved method of estimating caries attack rates in dental clinical trials. Presented at Annual Meeting of I.A.D.R., Houston, Texas, March 1969.

Serial No. NIDR-37 (68)
1. Biometry and Field Investigations Br.
2. Biometry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A:

Project Title: Evaluation of Reproducibility of Treatment Priority Index (TPI)

Previous Serial Number: None

Principal Investigator: Dr. R. A. Frew

Other Investigators: Dr. J. P. Carlos, Miss J. A. Brunelle

Cooperating Units: Division of Health Examination Statistics, National Center for Health Statistics

Man Years:

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

Project Description

Objectives:

To determine the reproducibility of the TPI for the assessment of occlusal deviations.

Methods Employed:

Five examiners (three dentists, a dental hygienist, and one non-dentist) performed replicate examinations on 100 sets of articulated dental models. One two-hour training session preceded the examinations. Examinations were conducted on an individual, unassisted basis and examiners were asked to complete their replicate examinations within one week.

The nine occlusal relationships described by Grainger were scored for each set of models by each examiner. Raw scores were weighted and summed for individual TPI and the mean TPI was calculated for each examination. Individual TPI scores were also ranked for each examination.

Part A (continued)

Inter and intra-examiner comparisons of scores and rankings were made utilizing significance testing for differences in means, product moment correlations, Spearman's rank correlation coefficient and Kendall's multiple rank correlation coefficient.

Major Findings:

Preliminary tabulations and analysis of the data indicate that the TPI is a reproducible measurement of occlusal deviations under the conditions operant in this study. Results obtained compare favorably with the reproducibility of other indices developed to quantitatively assess oral conditions.

Significance to Dental Research:

The TPI has been proposed as an index of malocclusion suitable for use by epidemiologists, public health programmers, and clinicians. Previous studies have shown that TPI scores correlate as well with clinical judgments as do replicate clinical judgments.

Preliminary results of this study indicate that the TPI is a reproducible quantitative assessment of occlusal deviations which can be utilized by dentists lacking specialty training and non-professionals, following adequate training.

Proposed Course of Project:

1. Complete tabulation and analysis of data on reproducibility of TPI.

2. Identify factor (s) contributing to TPI variance and attempt modification and or elimination of these items.

3. Identify modifications in data requirements which will enhance TPI as survey technique.

4. Report major findings.

Part B: Not included.

Serial No. NIDR-38 (68)
1. Biometry and Field Investigations Br.
2. Biometry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A:

Project Title: Similarity Analysis of Occlusal Relationships

Previous Serial Number: None

Principal Investigator: Dr. J. P. Carlos

Other Investigators: Dr. R. A. Frew

Cooperating Units: None

Man Years:

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

Project Description

Objectives:

To investigate the interrelationships of descriptive features of malocclusion using multivariate techniques and cluster analysis in order to determine those features which are most useful in classifying malocclusion.

Methods Employed:

Eighteen measurements of occlusal relationships were collected from each of 180 sets of interdigitated dental models. The models were randomly selected from a sample of approximately 1000 16-year-old high school students with no previous orthodontic treatment residing in the State of New York.

The data will be examined utilizing cluster or similarity analysis to identify those features which allow the best classification of occlusion in the study population. Identification of such features would permit their use in a severity index rating for malocclusion.

Part A (continued)

Major Findings: The study is in progress.

Significance to Dental Research:

Clinical judgment of the occlusal state is largely a subjective assessment. Numerous indices have been proposed to provide objective measurement of occlusal status. Most of these are a scoring and weighting of descriptive classifications developed from Angle's method. These indices have not proved adequate to permit selection of class boundaries on the scale of malocclusion with acceptable levels of discrimination.

This study of eighteen occlusal features will proceed with no prior assumptions concerning the distribution in the population of any one or combinations of the variables. An attempt will be made to identify an attribute or group of attributes which will permit the separation or grouping of individuals in a manner more definitive than current methods.

Proposed Course of the Project:

If an attribute or group of attributes which provide improved discrimination and show little independent variation can be identified from the attributes analyzed, an improved method for the assessment of malocclusion can be developed.

Part B: Not Included.

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 although shortage of man power and space as well as problems with equipment have made it difficult to pursue some of our goals with the necessary efficiency. Many of these deficiencies, however, are being corrected, thus ensuring a better utilization of our resources in the coming year.

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 efforts which have involved the use of the electron microscope and microradiographic methods are described under this heading. Many of the projects represent a continuation of previous studies although in several instances the investigations have progressed to new levels of refinement.

Research into the fine structure of the main sensory trigeminal nucleus has centered on the identification of the trigeminal primary afferent fibers and their relationship to the neurons in the nucleus as well as to axons projecting to the nucleus from the somatosensory area of the cerebral cortex. The demonstration of participation by the trigeminal primary afferents in axo-axonic synapses provides a fine-structural basis for recent neurophysiological findings, which have indicated reciprocal synaptic relationship between the primary afferents and the cerebral cortex. The significance of these connections is that impulses arriving via one pathway could diminish the effect of impulses arriving via the other pathway on the neurons of the nucleus. In addition the synaptic relationships between the trigeminal primary afferents and the dendrites and cell bodies in the nucleus appears to explain the rather strict somatotopic organization which characterizes the trigeminal brainstem nuclear complex.

Previous research by members of the staff into cell secretion has dealt primarily with cells which secrete the organic building blocks of mineralized tissues. The initiation this year of a project concerned with the fine structure of von Ebner's gland is considered a first step in the development of a program area dealing with the secretory mechanisms of exocrine glands, especially those which contribute their secretory product to the oral environment. Von Ebner's gland is one of the minor salivary glands, located in the tongue, which possibly plays a role in the sense of taste.

Ultrastructurally it resembles other serozymogenic glands and it appears that formation and discharge of secretory granules follow much the same pattern as that of the parotid gland as well as of the exocrine pancreas. This similarity as well as certain dissimilarities which have appeared in response to various stimuli make von Ebner's gland a very suitable object for continued studies. It is hoped that these will aid in clarifying many poorly understood aspects of the secretory process and help uncover some of the physical and chemical phenomena involved in the gustatory process.

In the program area of microbial morphology several new projects have evolved, all of which have been oriented toward the study of oral microorganisms and their potential for colonization on tooth surfaces. Of special interest has been the demonstration, for the first time, that at least one Gram negative oral bacterium will form plaque in a symbiotic relationship with known plaque formers such as diphtheroidal organisms. A presence of large numbers of Gram negative microorganisms in dental plaque suggests the possibility that the endotoxic LPS they produce plays a role in effecting or aggravating some of the tissue changes characteristic of periodontal disease.

It should be noted, however, that in no instance did the Gram negative organism tested initiate plaque formation. This finding stresses the importance of gaining additional insight into the conditions which underlie the formation of initial or primary plaque by oral diphteroids as was the objective of several other studies. The ability of these organisms to store carbohydrate intracellularly is being investigated, as is the possible role of the cell wall and cell membrane in synthesis of polysaccharide for extra- and intracellular use. Attempts at demonstrating a relationship between plaque forming ability and synthesis of extracellular dextran or levan have been negative. Instead, it appears that the composition of the extracellular products relevant to the process of adhesion is complex and variable.

Several of the studies which involve the use of the electron microscope represent collaborative efforts with other investigators. One such project has been the morphological mapping of 3 peptides isolated from the $\alpha 1$ chain of collagen by cyanogen bromide cleavage. SLS pieces formed from the 3 peptides exhibited a sufficiently well defined arrangement of bands to identify their relative position in the parent $\alpha 1$ chain. Although a distribution of the 8 peptides which result from cyanogen bromide cleavage has been proposed on basis of biochemical considerations, the morphological findings provide more accurate data.

Another collaborative project has been concerned with morphological alternations of tropocollagen interacted with human tissue collagenase. SLS prepared from such altered tropocollagen revealed that the collagenase, whether derived from leukocytes, bone or gingiva, cleaves the molecule into one-and three-quarter pieces, which subsequently become susceptible to hydrolysis by other proteases.

Histochemistry Section

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, (2) synovia from patients with rheumatoid activity, and (3) inflamed and or hyperplastic gingivae. The amount of collagenase detected in culture fluids of synovia correlated directly with the clinical severity of the disease.

A specific collagenase was detected in the granules of polymorphonuclear leukocytes from the peripheral blood of man, and collagenase has been detected in culture fluids of cartilage and bones of man. The enzyme requires calcium, and to a lesser extent sodium for activity. It is completely inhibited by EDTA and cysteine. Collagenase from synovial tissues in inhibited by serum whereas the enzyme derived from white blood cells is not. The enzyme is active over a broad range of pH from 7-9, with scant activity below pH 6. Electron microscopic and acrylamide gel studies indicate that collagenase from man divides the collagen molecule into two pieces approximately 3/4 and 1/4 in length, an action analogous to that described for collagenase derived from tadpole sources, but dissimilar to that described for bacterial collagenase.

Studies on hyaluronidase involved detection and description of the enzyme derived from alveolar macrophages of rabbits, and gingivae of man. The enzyme has a pH optima of 3.9 and thereby differs from that derived from oral microorganisms. The detection of hyaluronidase in various tissues aids in an explanation of 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 energy production, fatty acid, carbohydrate and mucopolysaccharide metabolism include the phosphatases, the esterases, numerous dehydrogenases, the glycosidases and the sulfatases. Esterases, glycosidases and sulfatases appeared to be correlated with cellular activity associated with active remodelling and bone resorption. The bulk of non-specific esterase of rat jaws was Type B with a small portion of Type C.

Crystal Chemistry Section

The Crystal Chemistry Section's long standing interest in synthetic calcium phosphates received added emphasis this past year with attention focussed primarily on the non-crystalline form of these compounds. The interest in this amorphous form of calcium phosphate stems from the finding that an amorphous analogue to this compound comprizes a major fraction of the mineral investing hard tissue. Unfortunately, direct study on in vivo amorphous calcium phosphate has proved to be difficult with generally inconclusive results. Consequently it has not been definitively demonstrated whether this in vivo material is a unique chemical entity, or is merely a cryptocrystalline form of hydroxyapatite. Current efforts are directed toward studies on synthetically prepared amorphous calcium phosphate with the expectation that some insight into the structural state of the in vivo analogue will be possible from these studies.

The present program is designed to more clearly define the snythetic amorphous salt through study of its thermochemical properties. Data thus obtained during the past year have shown that the synthetically prepared non-crystalline salt is most probably a hydrated tri-calcium phosphate, $Ca_3(PO_4)_2 \cdot 3H_2O$, suggesting that the amorphous component of hard tissue mineral is not a cryptocrystalline form of hydroxyapatite, but is chemically and structurally a distinct and separate phase.

A second line of investigation in the area of calcium phosphate chemistry was the initiation this past year of a study to re-evaluate the applicability of x-ray line broadening methods to the analysis of crystal size and perfection in bone and dentin hydroxyapatite. The need for this re-evaluation is apparent from the wide variation in reported values for these two parameters in studies using x-ray diffraction methods. The re-evaluation study has considerable relevance in that x-ray line broadening analysis is potentially one of the most powerful techniques available for the study of crystalline size and perfection in biological apatite. This study is still in its initial stages of development.

Another area of continuing interest to the section is infrared absorption spectrophotometry of hydroxyapatite and related compounds. An important advancement in this section's ability to study the IR spectra of hydroxyapatite has been the development of a variable temperature specimen holder permitting the spectra of hydroxyapatite samples to be investigated from $-185^{\circ}C$ to $250^{\circ}C$. With this unit it was possible to demonstrate OH $\cdots$F interactions in synthetic fluorhydroxyapatite. The importance of these IR observations is that they provide an experimental approach to solving the long-standing question of how relatively small amounts of fluoride suppress solubility and inhibit diffusion of impurities in apatites. Such information would almost certainly provide a rational explanation for the caries-inhibitory effect of fluoride ion in teeth.

This section continues to be engaged in collaborative studies with investigators in other laboratories. In one such project, a previous study into

the effect of covalent cross-linking on the crystallographic properties of bone and soft tissue collagen was continued. This year's research more clearly delineated the manner by which intermolecular cross-links protect bone collagen from irreversible denaturation. It was demonstrated that the cross-links in bone collagen exert a laterally directed restraining force between adjacent molecules, restricting their movement during denaturation. This restriction in mobility insures that original chain interrelations are not irretrievably lost, thereby preserving the ability of bone collagen to restore native structure.

In a continuation of another collaborative project on biofibers, it was demonstrated by x-ray diffraction that the cross-β pleated sheet configuration of fibrous amyloid protein was a property possessed by the individual molecules and was not the result of their association into fibrous arrays.

The results from a collaborative study on the formation of sulfur by the Chromatium genus of bacteria showed that this formation is marked by the successive appearance of three different allotropes of elemental sulfur. Initially, the sulfur appeared in the form of liquid-like globules which subsequently crystallized into an intermediate solid phase whose crystalline properties had not been previously described. This intermediate phase proved to be unstable and recrystallized slowly into stable orthorhombic sulfur. These events reflect the extreme rapidity by which sulfur is generated from the oxidative metabolism of sulfide by Chromatium. These studies on sulfur metabolism in Chromatium may provide some important clues into the mechanism by which non-crystalline calcium phosphate, also an inherently unstable phase, can be formed in hard tissue.

Experimental Pathology

This year's studies as were those of the preceding years have been aimed at gaining greater insights into the pathogenic potential of dento-bacterial plaques and into methods for controlling such deposits and their toxic products. In addition to laboratory experiments, the studies have included, for the first time, the testing of plaque control methods in human subjects.

The data obtained from laboratory experiments have revealed that pleomorphic diphteroids isolated from rats can induce periodontal lesions in hamsters and rats. In contrast, similar isolates from humans have not been pathogenic in either of these species. If the pathogens or pathogenic components which contribute to periodontal pathosis in humans can be found, more effective plaque control measures can be developed.

The relationship between cervico-radicular plaque infections and periodontal pathosis has been explored in mongoloid patients. The possible disclosure of plaque infection as an essential component will open the way for a more effective treatment of these patients as well as of other handicapped persons.

Laboratory findings have established that adhesion of caries conducive streptococci is somewhat dependent upon the formation of dextran from sucrose. The ability of the enzyme dextranase to disperse such deposits was tested in human volunteers. The initial tests which utilized a dextranase containing mouthwash were only partially successful. Presently the effectiveness of different methods of enzyme application is being explored.

These initial clinical studies show that there is an urgent need for the development of better diagnostic methods for the disclosure of dental plaque infections. It is also necessary to develop better assays for evaluation of the therapeutic value of mechanical and chemical treatment measures.

The establishment of a complimentary clinical program as an extension of the laboratory efforts will in the words of former President Johnson "speed the application of research knowledge to patient care so as to turn otherwise hollow laboratory triumphs into health victories."

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Collaborative Projects and Training Activities

Previous Serial Number: NIDR-39

Principal Investigator: Dr. M. U. Nylen, Dr. H. A. Bladen

Other Investigators: See below

Cooperating Units: See below

Man Years:

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

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 the organic moiety in calcareous corpuscles in tape worms. Small pieces of larval Taenia tacniaformis and mechanically isolated corpuscles from the same tapeworm were fixed in glutaraldehyde prior to decalcification in EDTA. The tissue pieces and some of the corpuscles were embedded and sectioned for examination in the electron microscope. The remaining corpuscles were negatively stained with

phosphotungstic acid and examined whole. The latter procedure revealed that the major portion of the organic moiety is found in the membrane(s) surrounding the corpuscles. With Dr. T. von Brand, Laboratory of Tropical Disease, NIAID. To be completed and written up this year.

2. Electron microscopic studies of the accessory boring organ of molluscs. This past year the work has been concerned largely with preparing the morphological data for publication. The cytochemical studies which were initiated last year had to be postponed because of shortage of personnel. It is anticipated that the efforts will be resumed next year. With Dr. M. R. Carriker, Marine Biological Laboratory, Woods Hole, Massachusetts, and Dr. V. Provenza, Maryland University Dental School, Baltimore, Maryland.

3. Experimentally induced enamel defects (Previous serial number: NIDR-38(63)). Previous experiments in this laboratory and elsewhere have demonstrated that defective areas in the enamel may become labelled permanently by a later injection with one of the tetracyclines. Although the reason for this is not well understood, it may be due to features special to the defective areas. To explore this phenomenon further a number of additional experiments were set up in which the interval between the first and second injection and the dose of the latter were varied. The shortage of personnel has prevented processing of this material, but it is expected that the effort will be resumed next year. With Dr. K.-A. Omnell and Dr. C.-G. Lofgren, School of Dentistry, Malmo, Sweden.

4. Morphological mapping of peptides isolated from the α 1 polypeptide chain of collagen. Three different peptides were isolated from the α 1 chain of collagen by cyanogen bromide cleavage. These were treated with ATP to form Segmented Long Spacings (SLS) and negatively stained with PTA. The structural integrity, i.e., the arrangement of bands at various intervals, along peptides CB6 and CB8 was precise enough to allow exact placement of these peptides in SLS prepared from intact α 1 polypeptides. The position of peptide CB7 was also located in the parent molecule. These three peptides comprise 65% of the α 1 polypeptide chain. During the last several months, more information was obtained concerning the mode of formation of SLS patterns in addition to more precise micrographs of the three peptides. Due to alterations in priorities, no further work in this area is anticipated. With Dr. Karl Piez, Laboratory of Biochemistry.

5. Morphological alteration of tropocollagen by human tissue collagenase. Collagenase was prepared from human granulocytic leukocytes as well as from human bone and gingival tissues. After reaction with solubilized collagen (Tropocollogen), Segmented Long Spacings (SLS) were formed by the addition of ATP. These were negatively stained and observed in the electron microscope. Pieces, one-quarter and three-quarter the original length of SLS were observed which indicate that the enzyme cleaves the tropocollagen one-quarter of the length from the carboxy terminal end

of the molecule. It is suggested that after collagenase activity, the fragments become susceptible to hydrolysis by other proteases. With Dr. G. Lazarus, and Dr. H. Fullmer. To be continued.

Training Activities:

Dr. Lenore Disher, School of Dentistry, University of California, San Francisco Medical Center and a U.S.P.H.S. post-doctoral fellow, was a guest worker in the laboratory until January 31, 1969. During this period she received training in electron microscopy and associated techniques.

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., Churchwell, F. K. and Stites, E.: Further observation on cestode calcareous corpuscles. Phosphate relationships, crystallization patterns, and variations in size and shape. Exptl. Parasitol. - in press.

2. Nylen, M. U. and Grupe, H. E.: Ultrastructure of epithelial cells in human periodontal explants. J. Periodont. Research - in press.

3. Nylen, M. U., Provenza, D. V. and Carriker, M. R.: Fine structure of the accessory boring organ of the gastropod Urosalpinx. Am. Zool. - accepted for publication.

4. Lofgren, C.-G., Omnell, K.-Å. and Nylen, M. U.: Effect of intraperitoneal injections of tetracycline hydrochloride and oxytetracycline on forming enamel of rat incisors. Calc. Tissue Research. 2:145-156, 1968.

5. Piez, K. A., Bladen, H. A., Bornstein, P., Butler, W. B., Kang, A. H., Lane, J. M. and Miller, E. J.: Studies on the chemistry of collagen utilizing cyanogen bromide cleavage. Brookhaven Symp. Biol. 2:345-357, 1968.

6. Lazarus, G. S., Daniels, J. R., Brown, R. S., Bladen, H. A. and Fullmer, H. M.: Degradation of Collagen by a human granulocyte collagenolytic system. J. Clin. Invest. 47:2622-2629, 1968.

7. Fullmer, H. M., Gibson, W. A., Lazarus, G. S., Bladen, H. A. and Whedon, L. A.: The origin of collagenase in periodontal tissues of man. J. Dent. Res. - in press.

8. Glenner, G. G. and Bladen, H. A.: The relationship of the two fiber types characteristic of human amyloid deposits. Proc. Symp. Amyloidosis, Groningen, 1967. Excerpta Med., Amsterdam, 1968.

Serial No. NIDR-40 (66)
1. Histology and Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Fine Structural Studies of the Trigeminal Brainstem Nuclei

Previous Serial Number: NIDR-42

Principal Investigator: Dr. Stephen Gobel

Other Investigators: Dr. R. Dubner, Dr. B. J. Sessle, Neural Mechanisms Section, NIDR

Cooperating Units: None

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives:

A. To continue studies of the main sensory nucleus in the following directions: (1) By means of surgical interruption and subsequent axonal degeneration of afferent pathways entering this nucleus, identify and study the distribution of: a. the primary afferent fibers of the trigeminal nerve; b. axons projecting to this nucleus from the somatosensory areas of the cerebral cortex. (2) To correlate and explain recent neurophysiological findings, such as presynaptic depolarization, in terms of the fine structural organization of this nucleus.

B. To extend fine structural studies to nucleus caudalis, the layered caudal end of the brainstem trigeminal nuclear complex in order to: (1) determine the normal morphological characteristics of the neurons and glial cells comprising this nucleus; (2) determine the normal synaptic relationships between the neurons of this nucleus and the primary afferent trigeminal axons.

Methods:

A procedure has been developed in which excellent preservation of adult cats' brainstems can be achieved consistently. The procedure essentially involves perfusing solutions of osmium tetroxide under pressure through the vertebral arteries. This is achieved by tieing the cannula conveying the perfusate into the ascending aorta and ligating the descending aorta, common carotid and axillary arteries. After routine dehydration and embedment in an epoxy resin, sections through the trigeminal brainstem nuclei are studied by light and electron microscopy.

In auxiliary light microscopical techniques, formalin fixed material was impregnated with silver salts. In one technique (Rapid Golgi method), thick sections (100 u) were used to study the type and distribution of neurons comprising the trigeminal brainstem nuclei as well as the branching pattern and dispersion of their dendrites. Other silver impregnation techniques (Nauta and Fink-Heimer methods) were used in conjunction with axonal degeneration studies to display axonal projections from the cerebral cortex to trigeminal brainstem nuclei. The latter techniques provide information with respect to the magnitude and location of the axonal endings in question and help guide the electron microscopical studies.

Major Findings:

The main sensory trigeminal nucleus is a semicircular mass of neurons, glial cells and neuropil approximately 1 mm. by 2 mm. The dendritic trees of the neurons consist of primary and secondary branches which in turn contain numerous spines. The dendritic branches of a single neuron while sparse in number are often long and may traverse broad expanses of the nucleus. Consequently in a given area of the nucleus one encounters dendrites of many neurons although the dendritic representation of any one neuron is meager.

The primary afferent trigeminal axons or sensory root of the trigeminal nerve on entering the main sensory nucleus form a series of large boutons (synaptic segments) which synapse on dendrites and neuronal cell bodies. The boutons of the primary afferent axons frequently form the core of complex structures called glomeruli, in which the central or primary afferent bouton is surrounded by alternating smaller axonal (peripheral) boutons and dendrites. Both central and peripheral boutons synapse on the dendrites.

Within the glomerulus, a primary afferent axon may synapse on dendrites of several neurons, and axo-axonic synapses are found between central and peripheral boutons. Axo-axonic synapses occur in three locations: (1) between the outer

surfaces of these boutons, (2) on evaginations of central boutons which project into peripheral boutons and (3) on evaginations of peripheral boutons which project into central boutons. In many instances two axo-axonic synapses have been found between central and peripheral boutons.

These observations suggest the existence of reciprocal presynaptic depolarization between corticofugal and primary afferent trigeminal axons. Recent physiologic experiments have demonstrated that corticofugal axons can indeed be depolarized by stimulation of primary afferent trigeminal axons. Consequently, it appears that corticofugal axons to the main sensory nucleus and the primary and trigeminal primary afferents are mutually antagonistic in the sense that each is capable of preventing impulses arriving via the opposite pathway from reaching the neurons of the main sensory nucleus. This kind of "presynaptic" or axonal modification of transmitted information relating to events taking place in the cerebrum and the external environment is only one facet of the complex processing of information which occurs continuously in the trigeminal system.

It is well known that the brainstem trigeminal nuclear complex is characterized, with few exceptions, by a rather strict somatotopic organization, i.e., neurons located in dorsal parts of the nucleus have receptive fields limited to mandibular regions of the face whereas the receptive field of neurons in ventral regions are confined to ophthalmic portions of the face. A medio-lateral somatotopy also exists with perioral receptive fields found for cells located in the medial part of the nucleus. The dorsal, middle and ventral parts of the nucleus receive trigeminal axons from the mandibular, maxillary and ophthalmic divisions, respectively.

In apparent contradiction to this somatotopic arrangement, Golgi preparations have revealed that the dendrites of many cells in the main sensory nucleus traverse broad expanses of the nucleus mediolaterally and dorsoventrally. Such cells undoubtedly are in contact with primary trigeminal afferents from more than one division. A detailed analysis of the mode of synapse of the primary trigeminal afferent fibers with the neurons of this nucleus has reconciled this apparent discrepancy between morphologic and physiologic observations. The important observations are briefly summarized as follows. Boutons of the primary trigeminal afferents from circular synaptic plaques .3 - .4 u in diameter irrespective of their location. The primary afferents merely "cross" the thin secondary dendrites making contact through single boutons whereas they often "climb" along the cell body and the thicker basal dendrites forming multiple contacts through two or more boutons. The area of contact between a primary afferent bouton and the cell

body and thick basal dendrites is relatively large in comparison to that between the primary afferent bouton and the thin secondary dendrites. Thus trigeminal boutons in contact with cell bodies should be more effective in bringing a cell to threshold (firing) than those located on the fine secondary dendrites because of the greater density of synapses.

Significance to Dental Research:

The trigeminal nerve conveys information to the central nervous system 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 brainstem 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 brainstem nuclei 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 associated with speech, mastication, deglutition and turning of the head can be effected. An appreciation of the fine structural basis of the processing of information in the trigeminal brainstem nuclei is essential for comprehending oro-facial function.

Proposed Course of the Project:

Long range plans involve extending the fine structural studies to other parts of the trigeminal brainstem nuclei and the trigeminal motor nucleus.

Part B

Publications:

Gobel, S. and Dubner, R.: Axo-axonic synapses in the main sensory trigeminal nucleus. Experientia 24:1250-1251, 1968.

Gobel, S. and Dubner, R.: Fine structural studies of the main sensory trigeminal nucleus in the cat and rat. J. Comp. Neurol. (In press).

Serial No. NIDR-41 (68)
1. Histology and Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: The Fine Structure of von Ebner's Gland

Previous Serial Number: None

Principal Investigator: Dr. A. R. Hand

Other Investigators: None

Cooperating Units: None

Man Years:

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

Project Description:

Objectives:

To determine the fine structure and mode of secretion of von Ebner's gland, a minor salivary gland located beneath the circumvallate and foliate papillae of the tongue, and if possible, its role, if any, in the sense of taste.

Methods:

The structure of the gland is being studied with the electron microscope, and the light microscope as an adjunct. von Ebner's glands are obtained from untreated rats, rats fasted for 24-48 hours, and from rats stimulated to secrete saliva by fasting-refeeding or intraperitoneal injection of a parasympathomimetic (pilocarpine) or B-adrenergic (isoproterenol) drug. Tissues are fixed by vascular perfusion through the heart or by immersion in buffered aldehyde solutions, and are post-fixed in buffered osmium tetroxide, dehydrated in graded ethanols and embedded in epoxy resins. An attempt is also being made to determine the ultrastructural histochemistry of the gland. As yet, the lead phosphate method of Gomori for acid phosphatase has been the only reaction tried.

Major Findings:

The ultrastructure of von Ebner's gland has been found to be similar to other sero-zymogenic glands, i.e., the parotid gland and the exocrine pancreas. Its mode of discharge of secretory granules is also similar to that of the parotid and pancreas, and morphological evidence suggests that synthesis and transport of proteins within the cell follow the pattern established for the pancreas.

Numerous heterogeneous dense bodies, presumably related to lysosomes have been found in the cells of von Ebner's gland. They are consistently related to the Golgi complex, and it is felt that they may play a role in degradation of excessive or "exhausted" membranous material. Protein transport in secretory cells occur via "carrier" vesicles which bud off of the transitional elements of the endoplasmic reticulum and subsequently fuse with Golgi cisternae or with condensing vacuoles. Considering volume and surface area of these vesicles, they would contribute much more membranous material to the Golgi region than would leave it surrounding the formed secretory granules. Thus a considerable amount of membrane material would accumulate in the Golgi region; yet this is not seen in cells which have been functioning for some time (6-8 week old animals). Degradation of Golgi membranes within lysosomal bodies would be one way to dispose of the surplus membrane material. The final lysosomal breakdown products may be the source of fat droplets seen in the cells, and would provide a store of lipid for synthetic or energy requiring processes.

Another structure observed regularly in the cells of von Ebner's gland is a "coated" or "alveolate" vesicle ranging in size from 500 to 2000 Å in diameter. They are seen most often in the Golgi region and close to or in connection with the cell membrane at basal, lateral or secretory surfaces. In other cells, the coated vesicles have been shown to be involved in protein uptake from the extracellular space, and also possess hydrolytic enzyme activity. The function of these vesicles in von Ebner's gland is unknown, but it is felt that they may be involved in the apparently active membrane degradation process described previously.

Significance to Dental Research:

Von Ebner's gland is one of the minor salivary glands, and its secretions help to make up the oral environment. The study of a part of that environment will lead to a better understanding of the whole environment.

The sensation of taste has been much studied but little understood. The study of von Ebner's gland may help to uncover some of the physical and chemical phenomena involved in the gustatory process. In addition, the study of this gland can add to the present knowledge of secretory mechanisms. The interrelation of neural and hormonal actions on exocrine glands and the role of myoepithelium in the

secretory process are still poorly understood. The Golgi apparatus has been a center of controversy since its discovery, and its complete function is not yet understood. It is hoped that this study will contribute to the morphological basis for biochemical phenomena occurring in the cell.

Proposed Course of the Project:

The scope of the project will be broadened to include further cytochemical studies. Additional studies on stimulated secretion will be carried out, with an attempt to use taste substances applied locally to the circumvallate papilla as a stimulus. A comparison of chemical and physical fixation of tissues, using the freeze-etch technique, will be attempted. In addition, the findings obtained from von Ebner's gland will be compared to findings of similar experiments on the major salivary glands.

Part B

Not included

Serial No. NIDR-42 (68)
1. Histology and Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Studies of Plaque Development by Oral Microorganisms

Previous Serial Number: None

Principal Investigator: Dr. H. A. Bladen, Dr. G. J. Hageage, Dr. G. S. Scott

Other Investigators: Dr. J. Tanzer

Cooperating Units: None

Man Years:

Total: 3

Professional: 1 3/4

Other: 1 1/4

Project Description:

Objectives:

To define conditions and explore parameters concerning the formation of microbial plaque. The specific objectives are (1) to study the production of *in vitro* plaque by an oral Gram negative organism and (2) to determine the effect of carbohydrates on *in vitro* plaque formation by oral diphtheroids, (3) to investigate the intracellular accumulation of polysaccharide in oral filamentous diphtheroids and (4) to determine the role played by their cell wall and cell membrane in polysaccharide synthesis.

Methods Employed:

1. Sterile notched nichrome steel wires were mounted in #2 rubber stoppers and suspended in test tubes containing 10 ml of basal medium with 2% sucrose inoculated with any of nine filamentous diphtheroids. The wires were transferred daily to freshly inoculated sucrose media and the primary plaques which formed were graded after 7 days. The

wires were then transferred daily to lactate media inoculated with Veillonella, a Gram negative oral organism. After 10 days, the secondary plaques were graded and compared to the grades obtained for primary plaques. Gram stains were prepared from plaque surfaces as well as from various media. Optical and electron microscopy was performed on complete plaque (primary plus secondary) grown on Epon rods which had been fixed and embedded.

2. Ten strains of oral diphtheroids, six of which were isolated from hamsters and rats and shown to be associated with periodontal pathosis in rodents, were employed in this study. The oral diphtheroids were maintained by bi-weekly passage in fluid thioglycollate medium.

For plaque growth study the organisms were inoculated in test tubes containing 10 ml of the medium of Jordan, Fitzgerald, and Bowler to which various sterile carbohydrate solutions had been added to give the final concentration of 0.5 and 5.0%. Sugars employed in this study were dextrose, fructose, sucrose, raffinose, or starch. Sterile nichrome steel wires mounted in No. 2 rubber stoppers were placed in the various media and incubated. Each day for five consecutive days the wires on which plaque was growing were transferred into freshly inoculated tubes of broth. On the five succeeding days, they were transferred into tubes of uninoculated broth. Plaque growth was rated after the fifth and tenth day according to the method of McCabe, Keyes and Howell.

3. Oral filamentous diphtheroids were grown in media containing 1%, 2%, 5%, 10%, or 15% sucrose, harvested, and washed six times in distilled water. The dry weight of the organisms was determined and aliquots were assayed for polysaccharide content by the anthrone method. In addition, samples were fixed, dehydrated, and embedded for electron microscopy.

4. Standard protoplast producing media were made containing penicillin, sucrose, and salts. Oral filamentous diphtheroids were grown in these media and examined for protoplast formation by optical and electron microscopy.

Major Findings:

1. All the filamentous diphtheroids formed primary plaques. After 10 days incubation in Veillonella inoculated lactate media which retarded growth of the diphtheroids, the plaques usually increased from a grade of 2 to a grade of 5. That the increased plaque growth was due to Veillonella (secondary plaque) was borne out by various findings.

First of all, while the type of plaque formed by the diphtheroids varied in consistency, the secondary plaque usually appeared hard and rough. Further, controls, consisting of primary plaque incubated in uninoculated lactate medium as well as primary plaques incubated in lactate medium inoculated with the respective filamentous organisms forming the primary plaque, showed no increase in plaque size. In no

case did Veillonella initiate plaque formation on wires when incubated alone in either sucrose or lactate media. Finally Gram stains of the outer surface of the secondary plaque revealed essentially Gram negative diplococci.

Optical microscopy of toluidine blue stained thick sections of Epon embedded primary plus secondary plaque formed on Epon rods revealed that the plaque was composed of two morphologically distinct types of bacteria, one a filament, the other a small coccus. The small cocci appeared to form many tightly packed microcolonies within the matrix of the filamentous diphtheroids. Generally the diphtheroids rather than the cocci were adjacent to the Epon rod while the cocci formed the outermost layer of the plaque.

Electron microscopy of the plaque specimens confirmed the optical microscopy observations. In addition, it was noted that larger spaces were present between the diphtheroids than between the cocci. No structural entities were observed in these spaces.

2. All strains were found to form plaque in the broths tested. Of the strains studied, no evidence was obtained to indicate that acknowledged pathogens produced more plaque than those strains whose pathogenicity has yet to be established. Generally dextrose, fructose, sucrose, and raffinose containing broths supported higher levels of plaque formation than did basal medium with starch or basal medium without added carbohydrate. In general, 10 day plaque ratings were one to two grades higher than plaque ratings at 5 days. In the case of 5% starch, however, plaque ratings often showed a marked increase between day 5 and day 10 even though inoculation of fresh broth had been discontinued. This phenomenon suggests the selection of mutant micro-organisms having increased adhesiveness when grown in the presence of starch.

The in vitro plaques observed in this study varied in consistency and morphology, but no common correlation could be made between plaque morphology and type or concentration of carbohydrate incorporated in the growth medium.

3. Certain strains of oral diphtheroids accumulated anthrone positive polysaccharide intracellularly in increasing amounts as the sucrose contents in the medium increased. Electron microscopic observation revealed the presence of intracytoplasmic vacuoles of the type commonly associated with polysaccharide formation and/or storage. The vacuoles appeared to be located in the central portion of the organism rather than in association with the cell membrane as commonly seen in plaque forming streptococci.

4. At least one diphtheroid strain has transformed successfully into a protoplast. The plaque forming potential of these protoplasts is being tested.

Significance to Dental Research

Clarification of the manner in which oral organisms contribute to plaque formation is of paramount importance in understanding the mode of action of periodontal disease producing organisms.

1. Gram negative microorganisms produce endotoxic LPS which is deleterious to tissue. The presence of large numbers of these organisms in the mouth suggests a potential capability of tissue destruction. The demonstration of the plaque forming potential of one Gram negative bacterium in a symbiotic relationship with known plaque formers is a first step in determining the potential role of such organisms in periodontal disease.

2. The present observations suggest that the plaque forming ability of the diphtheroidal organisms tested cannot be simply ascribed to the synthesis of extracellular dextran or levan by these cells. The formation of plaques in the presence of various carbohydrates, as well as the variable consistency of these plaques indicate that the composition of extra-cellular products germane to the process of adhesion is likely to be complex and variable.

3. The ability of certain oral diphtheroidal organisms to accumulate intracellular polysaccharide in the presence of excess sugar may allow them to continue plaque formation after withdrawal of extracellular carbohydrate.

4. Determination of the role of the cell wall and cell membrane in polysaccharide synthesis will furnish data of importance in future considerations of possible means toward prevention or suppression of plaque formation by filamentous organisms.

Proposed Course of Projects:

1. This work is to be continued in an effort to define the contribution of Gram negative organisms to periodontal disease. Present plans call for *in vivo* as well as *in vitro* studies. These are to include a "morphological approach" to the plaque to tissue relationship.

2. Studies concerning (1) the fine structure of the different types of *in vitro* plaque formed in media incorporating various carbohydrates, (2) the isolation and characterization of the extracellular material formed during *in vitro* plaque formation and (3) the development of measures designed to inhibit formation of . . . or remove already formed . . . *in vitro* and *in vivo* plaques.

3. Present plans include relating chemical and morphological data on intracellular polysaccharide accumulation to the amount of excess sucrose and the duration of exposure. In addition depletion studies are planned to explore the ability of the polysaccharide laden organisms to continue plaque formation in the absence of extracellular sucrose.

4. Efforts to define the role of the cell wall and cell membrane of the filamentous oral organisms in polysaccharide synthesis will be continued.

Part B

Not included.

Serial No. NIDR-43 (66)
1. Histology and Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: The Relationship Between Function and Structure in Micro-organisms

Previous Serial Number: NIDR-44

Principal Investigator: Dr. G. J. Hageage

Other Investigators: Dr. H. A. Bladen, Dr. E. D. Eanes

Cooperating Units: Dr. R. L. Gherna (American Type Culture Collection)

Man Years:

Total: 1 3/4

Professional: 3/4

Other: 1

Project Description:

Objectives:

The studies involving morphofunctional relationships under investigation 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 objective of the present study are three fold: (1) To determine the site of sulfide oxidation in the photosynthetic bacterium Chromatium. (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 in Chromatium species.

Methods Employed:

Chromatium okenii and Chromatium wessei were grown in screw-capped bottles in a synthetic mineral medium containing Vitamin B_{12} and sodium sulfide. The cultures were incubated at 25 - 27°C with continuous illumination at 300 ft-c. Chromatium warmingii was grown in the same

manner but at an illumination of 50 ft-c during a 16 hour light cycle.

Cells to be examined by electron microscopy were either (1) negatively stained with phosphotungstic acid, (2) fixed, dehydrated, and embedded according to the procedure of Kellenberger prior to sectioning or (3) were glycerinated in 10% glycerin for 8 hours, frozen in liquid freon 22, and fractured according to the method of Steere.

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 either nitrogen-flushed distilled water or boiled distilled water. X-ray diffraction patterns of freshly isolated wet sulfur packed in 0.5 mm or 0.7 mm capillary tubes were taken with either a 57.3 mm or a 114.6 mm diameter Debye-Scherrer powder camera. Ni filtered copper radiation (λ = 1.542 Å) was employed as the x-ray source. The capillary-mounted wet sulfur specimens were examined by x-rays at irregular intervals up to 6 weeks.

Major Findings:

As previously reported thin sections and freeze-etched preparations of sulfide-fed cells revealed sulfur globules encompassed by an electron-dense border as well as by the same unit membrane (presumably the cytoplasmic membrane) that invaginates to form the chromatophores. This indicates that the sulfur globules are not intracellular per se but are actually outside the cytoplasm.

The x-ray diffraction diagrams of freshly isolated wet sulfur globules from C. wessei and C. okenii gave two broad and diffuse diffraction rings with maxima at about 3.6 Å and 5.2 Å. The 3.6 Å diffraction ring was always more prominent, being about twice as intense as the 5.2 ring. The sulfur pattern in x-ray diffraction diagrams from C. warmingii was similar but less well defined. This amorphous-like x-ray pattern from freshly isolated sulfur resembles closely the diffraction pattern of liquid sulfur which has two maxima at about 5 Å and 3.5 Å. When kept in a wet state, the sulfur globules slowly crystallize with a crystalline pattern appearing after 24 hours. This pattern is fully developed by 4 days. Although the interplanar d-spacings and the relative intensities of the diffraction lines have been calibrated, a positive identification of this crystalline phase from the x-ray pattern has not been obtained. The x-ray pattern does not match those reported for orthorhombic sulfur, monoclinic (β) sulfur, rhombohedral sulfur or insoluble sulfur. Nor do the observed d-spacings compare with d-spacings calculated from published unit cell data for monoclinic (α) sulfur, hexagonal sulfur, or fibrous sulfur. This crystalline allotrope of sulfur is metastable and slowly transforms into orthorhombic sulfur within six weeks. Vacuum drying the preparation accelerates the change into orthorhombic sulfur. Thus the formation of sulfur by Chromatium species is marked by the presence of three allotropes, (1) a liquid-like globule formed by the photooxidation of sulfides, (2) an

intermediate crystalline phase of limited stability, and (3) stable orthorhombic sulfur.

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 sulfide by members of the Thiorhodaceae will be continued. Studies concerning the nature of the sulfur formed from the photooxidation of sulfide have been completed and this portion of the project is being written up.

Part B

Not included.

Serial No. NIDR-44 (55)
1. Histology and Pathology
2. Histochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Studies of Human Collagenase

Previous Serial Number: NIDR-47

Principal Investigator: Dr. H. M. Fullmer

Other Investigators: Dr. W. A. Gibson, Dr. J. F. Goggins

Cooperating Units: Dr. G. S. Lazarus
Department of Dermatology
Massachusetts General Hospital
Boston, Massachusetts

Man Years:

Total:	4
Professional:	1
Other:	3

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 day for 7 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: Methods that have been found useful for purification of human collagenase are ammonium sulfate precipitation, electrophoresis with the curtain type Brinkman Model FF, and column chromatography with DEAE and agarose.

   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 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 been 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.

Collagenase production by synovial tissues in culture has been found to be directly related to the clinical severity of rheumatoid arthritis and to the cellularity of the tissues. Increased collagenase production was also correlated with increased vascularity of of the tissues.

Preliminary data from recent experiments indicates collagenase production by gingivae cultured in vitro is directly related to the clinical severity of periodontitis. The more active the periodontitis, the greater the production of collagenase.

Collagenase from polymorphonuclear leukocytes has been isolated, partially purified and characterized.

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. Goggins, J. F., and Fullmer, H. M.: Hyaluronidase activity of human gingivae. AMA Arch. Path. 85:272-274, 1968.

2. Lazarus, G. S., Brown, R. S., Daniels, J. R., and Fullmer, H. M.: Human granulocyte collagenase. Science 159:1443-1445, 1968.

3. Fullmer, H. M., Lazarus, G. S., Stam, A. C., Jr., and Gibson, W. A.: Collagenase in neuromuscular diseases. In Norris, F. H., Kurtland, L. T. (Ed.): Motor Neuron Diseases: Research on Amyotrophic Lateral Sclerosis and Related Disorders. New York, Grune & Stratton, 1969, pp. 242-244.

4. Lazarus, G. S., Decker, J. L., Oliver, C. H., Daniels, J. R., Multz, C. V., and Fullmer, H. M.: Collagenolytic activity of synovium in rheumatoid arthritis. N. E. J. Med. 279:914-919, 1968.

5. Lazarus, G. S., Daniels, J. R., Brown, R. S., Bladen, H. A., and Fullmer, H. M.: Degradation of collagen by a human granulocyte collagenolytic system. J. Clin. Invest. 47:2622-2629, 1968.

6. Fullmer, H. M., Gibson, W. A., Lazarus, G. S., Bladen, H. A., and Whedon, K. A.: The origin of collagenase in periodontal tissues of man. J. Dent. Res. (In press).

7. Lazarus, G. S., and Fullmer, H. M.: Collagenase production by human dermis in vitro. J. Invest. Derm. (In press).

8. Fullmer, H. M., Gibson, W. A., Lazarus, G. S.: Collagenase studies of gingiva of man. Trans. 3rd Internation Congress of Oral Surgery. (In press).

9. Fullmer, H. M., and Lazarus, G. S.: Collagenase in bones of man. J. Cell Biol. (In press).

10. Fullmer, H. M.: A decalcification technic for enzyme preservation. In Finn, S. B. (Ed.): BIOLOGY OF THE DENTAL PULP ORGAN: A Symposium. Birmingham, Ala., University of Alabama Press, 1968, pp. 265-272.

Serial No. NIDR-45 (64)
1. Histology and Pathology
2. Histochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Histochemical and Chemical Studies of Connective Tissues

Previous Serial Number: NIDR-48

Principal Investigator: Dr. W. A. Gibson

Other Investigator: Dr. H. M. Fullmer, Dr. J. F. Goggins

Cooperating Units: Dr. L. Weiss, Dr. M. Neiders
Roswell Park Memorial Institute, Buffalo, N. Y.

Man Years:

Total: 2 1/2

Professional: 1

Other: 1 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:

Electrophoretic separations and identification of various enzymes and their molecular variants are being performed to supplement information from histo-chemical studies as well as to evaluate histochemical methods.

C. Cell and Tissues 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 for 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.

D. Under research contract PH-43-67-1460 with Roswell Park Memorial Institute Drs. Leonard Weiss and Mirdza Neiders are investigating a "Biophysical Approach to Epithelial Attachment and Detachment." Expenditures in fiscal year 1968 were $7,150. The contract was initiated June 19, 1967 and will continue until June 18, 1969 at which time the project will be transferred to grant support. Methods employed include: isolation and growth of several types of cells including human gingival cells in suspension cultures, electrokinetic determination of surface charges of cells, cementum and enamel and attachment and detachment studies of cells on various surfaces before and after treatment with hydrolytic enzymes and lysosomal stabilizers and labilizers.

Major Findings:

A. In the continuing study of the histochemistry of the rat periodontal ligament aryl sulfatase activity was correlated with the cellular activity associated with bone resorption. In sections stained with the simultaneous coupling method of Wooksman and Hartrodt only osteoclasts and perivascular cells in areas of bone resorption were found to have appreciable amounts of activity.

The type of aryl sulfatase activity demonstrated appeared not to be that of the classically described type I or type II. Whether this indicates a new type of aryl sulfatase is subject for further study.

B. β-N-acetylglucosaminidase has been isolated from rat livers and partially purified. The purified enzyme will be used in a fluorescent antibody study to confirm routine histochemical localization of the enzyme in the rat periodontal ligament.

Aryl sulfatase has also been isolated from rat livers and the purified enzyme will be used in a study to determine the type of aryl sulfatase found in the rat periodontal ligament.

C. Suspension cultures of human gingival epithelial cells yield uniform viable cells but the life span of such cells is short since they rapidly undergo keratinization. Plate cultures from such suspensions are being tried along with various media to produce a uniform cell culture of reasonably long life span.

D. The work progress to date on NIDR contract no. PH 43-67-1430 under the direction of Dr. Weiss has been quite substantial. Several aspects of the work are particularly noteworthy. The method devised by Dr. Neiders for establishing primary cultures of human gingival epithelial cells has been adapted for certain work in our own laboratory.

The finding that keratinization proceeds in such cells in spinner cultures is exciting and the method could prove to be a new approach to studying the mechanisms involved in keratinization.

It has been known for some time that the facility of reattachment of gingival tissues to freshly exposed enamel and cementum is different than that to surfaces that had been exposed for any length of time to the oral environment. Results from the electro-kinetic studies definitely show differences in the charges of exposed and nonexposed surfaces. Continued studies along these lines as well as the characterization of gingival cell surfaces could lead to changes in the fundamental concepts of the periodontium, periodontal disease and treatment procedures.

The method devised by Dr. Neiders for growing gingival cells on flat tooth surfaces has proven to be quite successful and should lead to a further understanding of the definitive attachment mechanisms.

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., Harrison, E. T. and Fullmer, H. M.: Multiple esterases of the rat jaw. Archs. Oral Biol. 13:1279-1280, 1968.

2. Gibson, W. A. and Fullmer, H. M.: Histochemistry of the periodontal ligament. IV. The glycosidases. J. Perio-Periodont. (in press).

Serial No. NIDR-46 (66)
1. Histology and Pathology
2. Histochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Histochemical and Chemical Studies of Connective Tissues and Teeth

Previous Serial Number: NIDR-49

Principal Investigator: Dr. J. F. Goggins

Other Investigators: Dr. H. M. Fullmer, Dr. J. M. Tanzer

Cooperating Units: None

Man Years:

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

Project Description:

Objectives:

A. To determine certain metabolic parameters of normal and diseased tissues and to use and develop techniques for the detection and assay of some of the enzymes involved.

B. To relate these enzymes to the tissue physiology or pathology by the development and application of qualitative and quantitative histochemical and biochemical methods.

Methods Employed:

A. Biochemical

Standard spectrophotometric and fluorimetric assays for enzyme activities in various tissue homogenates are utilized in order to establish baseline data such as total tissue activity and optimal incubation conditions for a particular enzyme. These data serve as a reference for the adaption of the methods to a micro scale for quantitative histochemistry.

B. Slide Histochemical

Since enzymes are the substances of current interest, tissue specimens are handled in a manner to minimize 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.

C. Quantitative Histochemical

Ultramicro analytical procedures such as those devised by Lowry are being adapted for the study of enzymes involved in mucopolysaccharide metabolism in various small areas of normal and diseased human gingiva. β-Glucuronidase activity is being measured by a microfluorimetric method currently under development. The method utilizes napthol-AS-BI-β-D-glucuronide as substrate and activity is assayed by measurement of the fluorescence of the napthol AS-BI liberated by enzyme action on the substrate. Quantitative comparisons of enzyme activities are made in relation to cell types and function as well as physiological or pathological state of the particular cells or tissues.

Major Findings:

A. Examination of sections of human gingiva stained for β-glucuronidase and N-acetylhexosaminidase activity revealed a similar distribution for both enzymes in the gingival connective tissues. Marked activity was noted in fibroblasts and macrophages, particularly in areas surrounding round cell infiltrates. The round cell infiltrates , on the other hand, showed minimal activity. The reaction products were intracellular and generally showed a perinuclear distribution.

The β-glucuronidase activity of human gingiva has a pH optimum of 5 and an apparent Km for napthol AS-BI-β-D-glucuronide of 5×10^{-4}M. The activity is linear with time for at least one hour and is proportional to enzyme concentration. The principal disadvantage of the substrate is that high substrate concentration inhibit enzyme activity which necessitates working with substrate concentrations near the Km value. However, the method is sufficiently sensitive to detect β-glucuronidase activity in 0.2 ug samples of gingival connective tissue.

B. The use of droplet incubation within wells filled with light mineral oil has been adapted for incubations for micro assays of enzyme activity. It was found that droplet incubation offered the following advantages over standard test tube incubations: 1) incubations can be conducted in very small volumes, 2) evaporation is well controlled, 3) manipulative procedures are simplified, and 4) there is no loss of enzyme activity if the enzymes are adequately protected from inactivation at the oil-water interface by the addition of 0.1% non-reactive protein.

C. A method of calibrating micropipets was developed which utilizes a comparison of radioactivity of delivered aliquots of a radioactive solution from standard and unknown pipets. It was found that the method is equally precise as the colorimetric method and is much less time consuming because automated counting equipment can be used.

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:

Qualitative and quantitative histochemical investigations of enzymes in connective tissues will continue. A particular field of study will be the correlation of data from utilization of quantitative microchemical methods to obtain reliable information about the metabolic status of small regions. Qualitative assays suggest wide variations of enzyme activity in various regions of gingiva, for example, depending on the function of the cells at any particular time. In addition, efforts are in progress to adapt micro assays for DNA determinations for use with these tissues. With this, enzyme activities in different areas of tissue can be compared not only on the basis of dry weight of tissue but also on the basis of cell content.

Part B

Publications:

1. Goggins, J. F. and Gibson, W. A.: Histochemistry of viable frozen-stored human gingiva. J. Invest. Derm. 51:137-140, 1968.

2. Goggins, J. F., Lazarus, G. S. and Fullmer, H. M.: Hyaluronidase activity of alveolar macrophages. J. Histochem. Cytochem. 16:688-692, 1968.

3. Goggins, J. F. and Tanzer, J. M.: A rapid method for calibration of micropipets. Anal. Chem. Acta (In press).

Serial No. NIDR-47 (68)
1. Histology and Pathology
2. Histochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Purification of Rat Osteoclasts

Previous Serial Number: None

Principal Investigator: Dr. W. B. Riley, Jr.

Other Investigators: None

Cooperating Units: Dr. T. G. Pretlow, II, National Cancer Institute

Man Years:

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

Project Description:

Objectives:

A. To extract osteoclasts from rat femurs and purify them (i.e., separate them from other cells) by sedimentation in Ficoll density gradients.

B. To study the metabolism of osteoclasts under cell culture conditions. Of primary importance in determining an enzyme profile for osteoclasts will be qualitative and quantitative studies of specific collagenase activity. If such studies are fruitful in the rat, normal and diseased human bone will be studied in a similar fashion.

Methods Employed:

1. Density Gradient Centrifugation: Small aliquots of normal saline are flushed through the distal metaphyses of rat femurs, affording a saline-suspended mixture of approximately 0.1% osteoclasts and 99.9% other cells. Such a suspension is layered over the top of a linear density gradient of Ficoll

(sucrose polymer) in tissue culture medium. Optimal speed and duration of centrifugation are determined by the computer-integrated differential sedimentation equation. Fractions are collected, slides are made, and differential cell counts performed.

2. Optimal culture conditions have not yet been determined.

Major Findings

Preliminary results indicate that we have obtained aliquots of rat osteoclasts in 10-30% purity, which, in turn represents a 250-500 fold purification.

Significance to Dental Research

Little is known about the specific mechanisms of normal bone resorption at the cellular level. In addition, many human diseases are characterized by abnormal bone resorption. Although osteoclasts have long been implicated in both normal and abnormal resorptive processes, direct evidence of specific degradative enzyme production and elaboration is lacking. Such studies, however, would be possible under pure cell culture conditions.

Proposed Course of Project

There are two immediate goals:

1. To increase both the purity and the yield of the osteoclasts.
2. To determine optimal cell culture conditions for osteoclasts.

Part B

Publications

1. Riley, W. B., and Fullmer, H. M.: A brief review. Dermatology Digest (in press).

Serial No. NIDR-48 (68)
1. Histology and Pathology
2. Crystal Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Thermochemical Studies on Non-crystalline Calcium Phosphates

Previous Serial Number: None

Principal Investigator: Dr. E. D. Eanes

Other Investigators: None

Cooperating Units: None

Man Years:

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

Project Description:

Objectives:

The purpose of this project is to more fully define the structure and composition of non-crystalline calcium phosphates through the study of their thermochemical properties. The principal biological objective is to acquire from studies on synthetic counterparts a more complete understanding of the origin, nature, and function of the non-crystalline component of hard tissue mineral. An ancillary objective involves the development of a thermochemical technique to detect the presence of this amorphous mineral component in hard tissues. During the year covered by this report, the principal undertaking was to establish the basic thermal behavior of synthetically prepared non-crystalline calcium phosphates. This undertaking was initiated with two specific objectives in mind, (1) to determine the phase changes, and (2) to determine the compositional changes which occur upon igniting these materials.

Methods Employed:

The non-crystalline calcium phosphates were prepared by spontaneous precipitation from rapidly mixed alkaline solutions of soluble calcium and phosphate salts. All preparations were thoroughly washed and freeze-dried immediately following precipitation. Ignition of these materials were carried out between 500°C and 1100°C over time intervals ranging from less than one hour to over 96 hours. The reaction products following thermal treatment were studied by x-ray diffraction and chemical methods. X-ray diagrams were recorded photographically with Debye-Scherrer powder cameras and the structural identity of crystalline phases established by comparison with reference standards. Chemical analyses were employed to determine the calcium, orthophosphate, and pyrophosphate content of the preparations before heating and the products of ignition.

Major Findings:

Non-crystalline calcium phosphates prepared from aqueous solution are three component phases, chemically, in that they are compositionally definable in terms of CaO, P_2O_5, and H_2O. However, many formulae expressing their composition are possible within the framework of this ternary phase system. As examples, the non-crystalline calcium phosphates could be calcium deficient hydroxyapatites, lamellar intergrowth of octacalcium phosphate and hydroxyapatite, or hydrated tricalcium phesphates. An observed CaO/P_2O_5 molar ratio of 3 and an amorphous-like x-ray diffraction pattern do not exclude any of these compositional alternatives.

The thermochemical data does suggest, however, that the water component in these aqueous-prepared non-crystalline calcium phosphates retains its molecular identity. Three lines of thermal evidence support this conclusion. First, non-crystalline calcium phosphate can be observed by x-ray diffraction. Secondly, if the water component was split and existed in combination with the P_2O_5 component as acid phosphate, then the pyrophosphate level upon igniting non-crystalline calcium phosphate to just below its crystallization point (500°C) would be considerably higher than actually observed. Finally, the splitting of molecular water apparently can only occur when the non-crystalline calcium phosphate is allowed to interact with molecular water at elevated temperatures. When ignited in the presence of water vapor at 600°C, the non-crystalline calcium phosphate converts rapidly into calcium-deficient hydroxyapatite, an acid phosphate containing compound. But when the water vapor interaction is minimized at this temperature, α- and/or β-tricalcium phosphates are formed instead of hydroxyapatite. These tricalcium phosphate salts, furthermore, can be defined solely within the framework of the binary system $CaO-P_2O_5$.

The thermal data, then, strongly favors the hydrated tricalcium phosphate formula as the correct expression for the composition of non-crystalline calcium phosphate. It is the only formula of the three given in which the aqueous component is present entirely as water molecules. A weight loss of 15% upon dehydration suggests further that the exact composition of freeze-dried non-crystalline calcium phosphate is $Ca_3(PO_4)_2 \cdot 3H_2O$.

The thermal behavior of non-crystalline calcium phosphate in those ignition procedures which minimized water interactions was examined in some detail. Crystallization began at about 550°C. Up to this temperature, the non-crystalline features of the material are thermally stable. The first crystalline phase to appear was invariably β-tricalcium phosphate. Above 600°C, however, α-tricalcium phosphate was the favored ignition product even though β-tricalcium phosphate is normally the stable phase up to 1140°C. The emergence of unstable α-tricalcium phosphate suggests that the thermocrystallization of non-crystalline calcium phosphate is a kinetically rather than a thermodynamically controlled process. The transformation α-tricalcium phosphate to the β-form is very sluggish below 1100°C, however, and this would account for the apparent metastability of the α-tricalcium phosphate once formed.

This thermal behavior of anhydrous non-crystalline calcium phosphate is in direct contrast to that of calcium-deficient hydroxyapatite. This latter compound is thermally more stable and retains its structural identity up to 650°C before converting into tricalcium phosphate. Also the conversion products appear in inverse sequence to that found for the non-crystalline salt with the α-form appearing at lower ignition temperatures and the β-form at higher temperatures. This finding is further evidence that non-crystalline calcium phosphate is not a cryptocrystalline form of calcium-deficient hydroxyapatite, and strengthens the contention that non-crystalline calcium phosphate is a true tricalcium phosphate.

Significance to Dental Research:

Non-crystalline calcium phosphate is postulated to be a principal component of the mineral portion of bone and dentin. But because of its intimate association with hydroxyapatite and collagen, it has proved to be difficult to study the properties of this material *in vivo*. To develop guidelines along which meaningful *in vivo* studies of this material can be developed, a thorough physical and chemical study of its synthetic analogue has been undertaken. The thermal studies covered in this report strengthens the argument that the non-crystalline component of hard tissue mineral is non-apatitic in nature and is most probably a hydrated tricalcium phosphate. The importance of this finding is that bone and dentin mineral must be considered as being a two phase system and previous theories of calcification must be modified to take this consideration into account.

Proposed Course of Project:

It is hoped from the present understanding of the thermal properties of synthetic non-crystalline calcium phosphate that techniques can be developed to study the thermal behavior of *in vivo* non-crystalline calcium phosphate with particular emphasis on devising x-ray diffraction tests that will detect this phase in small quantities of hard tissues. The thermal behavior of synthetic non-crystalline calcium phosphate in the presence of pyrophosphate, magnesium, and carbonate will be studied. These three ions have been implicated most often in accounting for the unusual stability of *in vivo* non-crystalline calcium phosphate.

Part B

Not included.

Serial No. NIDR-49 (67)
1. Histology and Pathology
2. Crystal Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: X-ray Diffraction Studies on Fibrous Proteins

Previous Serial Number: NIDR-51

Principal Investigator: Dr. E. D. Eanes

Other Investigators: Dr. E. J. Miller

Cooperating Units: Dr. G. G. Glenner, NIAMD-LEP

Man Years:

Total: 1/2

Professional: 1/4

Other: 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 previously undertaken studies were continued 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 techniques employed were wide-angle and small-angle x-ray diffraction. Collagen was obtained from demineralized tibia of 3-week old chicks and from tail tendon of growing Sprague-Dawley rats. The collagen was examined dry or in contact with either distilled water, 0.5 M acetic acid, or 5 M guanidine-HCl (pH 7.0). Fibrous amyloid was obtained from the organs of human patients and experimental animals afflicted with amyloidosis. Both fresh tissue and purified fibrous extracts were studied.

Major Findings:

1. Collagen study: Previous work under this project established that bone collagen and rat tail tendon are essentially similar in their response to distilled water and acetic acid. There is no loss of helical structure in these solvents but lateral separation of adjacent molecular units occur. Likewise both collagens denature in 5 M guanidine · HCl. Only when the denaturing agent is removed do significant contrasts in molecular behavior occur. The bone collagen reestablishes native structure whereas the rat tail tendon remains denatured. These findings were interpreted in terms of the type of covalent cross-linking existing in the two collagens. The high degree of intermolecular cross-linking in bone collagen provides a number of fixed points restricting the movement of molecules during denaturation so as to allow for rapid restoration of native relationships between individual chains and molecules upon renaturation. Current studies have shed further insight into how the cross-links in bone collagen regulate this movement of molecules during denaturation. Rat tail tendon is devoid of intermolecular cross-links. It was found, however, that these fibers could retain the ability to renature if they are constrained from laterally swelling during denaturation. Allowing the tendon fibers to denature free of physical restraint or constraining them from axially shrinking during denaturation did not prevent irreversible disorder. Apparently, then, it is laterally imposed restraint that is important in preserving interchain relationships necessary for helical restoration. One can therefore picture the intermolecular cross-links in bone collagen exerting a laterally directed restraining force between adjacent molecules which prevent the latter from migrating apart to the point where original chain interrelations, both between and within the molecules, are irretrievably lost.

The small-angle diffraction pattern from bone collagen appears to be devoid of discrete diffraction maxima. This is in distinct contrast to the small angle pattern from rat tail tendon where discrete diffraction lines are its most distinctive feature. This contrast does not appear to be associated with the lower degree of fiber orientation in bone collagen. The exact origin of these diffraction differences, however, has not as yet been established.

2. Amyloid study: In collaboration with Dr. G. G. Glenner, NIAMD, studies on amyloid were continued. As previously reported, the diffraction pattern is of the cross-β type suggesting that the fiber is a pleated sheet structure formed by the amyloid molecules folding in a regular manner on themselves such that adjacent chain segments in each molecule are laterally arranged in an antiparallel manner. The current study has demonstrated that the pleated-sheet configuration is a property possessed by the individual molecules and is not the result of their association into fibrous arrays.

The small angle diffraction pattern from fibrous amyloid exhibited a pair of arcs at a d-spacing of 47 Å. The reflections are equatorially positioned and, therefore, represent diffraction features perpendicular to the fiber axis. The 47 Å spacing is presently interpreted as a measure of the lateral distance between adjacent fibers.

Significance to Dental Research:

1. Since collagen is an important component in all connective tissues including dentin and the periodontal membrane, 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 fetal 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 studies on collagen and amyloid will be continued but with greater emphasis on the small-angle x-ray diffraction properties of these fibrous proteins.

Part B

Publications:

1. Eanes, E. D., and Miller, E. J.: Effect of Covalent Cross-linking on the X-ray Diffraction Properties of Chick Bone and Rat Tail Tendon Collagens. Archs. Biochem. Biophys. 129:769-771, 1969.

2. Eanes, E. D., and Glenner, G. G.: X-ray Diffraction Studies on Amyloid Filaments. J. Histochem. Cytochem. 16:673-677, 1968.

3. Glenner, G. G., Keiser, H. R., Bladen, H. A., Cuatrecasas, P., Eanes, E. D., Ram, J. S., Kanfer, J. N., and DeLellis, R. A.: Amyloid. VI. A Comparison of Two Morphologic Components of Human Amyloid Deposits. J. Histochem. Cytochem.16:633-644, 1968.

Serial No. NIDR-50 (68)
1. Histology and Pathology
2. Crystal Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: X-ray Line Broadening Analysis of Synthetic and Biological Hydroxyapatites.

Previous Serial Number: None

Principal Investigator: Dr. D. R. Lundy

Other Investigators: Dr. E. D. Eanes

Cooperating Units: None

Man Years:

Total: 1/2

Professional: 1/2

Other: 0

Project Description:

Objectives:

The purpose of the study is to clarify the relative contributions of crystal size and internal crystal strain to the broadening of the x-ray diffraction profiles of hydroxyapatite. The information provided by this study is essential if x-ray diffraction procedures are to be used to elucidate the crystal size and degree of crystal imperfection of biological apatites.

Methods Employed:

Samples of synthetically prepared hydroxyapatite are being used as test materials. These samples are being prepared by a variety of synthetic processes to insure that the test materials will reflect a wide range of crystal sizes and degrees of chemical perfection.

Precision wide angle x-ray diffraction techniques are being employed to record, accurately, the principal diffraction features of these test materials. The diffraction line of greatest interest is the 002 reflection from hydroxyapatite. It is the only reflection free of overlap by adjacent lines, a feature essential for the proper application of numerical test procedures designed to separate the size and strain contributions from the total broadened profile.

Diffraction data is being analyzed by three line broadening procedures: (1) integral breadth analysis, (2) Fourier analysis, and (3) variance analysis. Each of these three methods give essentially independent estimates of crystal size and strain. Comparability of results, then, can be used as one test of the validity of the procedures. Computer programs have been developed to carry out the numerical computations involved in applying these analytical procedures to the experimental x-ray data. These programs have been checked out using data from theoretical curves and experimental curves from the literature.

Correlating the results from the x-ray line broadening analyses with the chemical history of the synthetically prepared hydroxyapatite test samples used will, hopefully, enable us to assess the influence of enviromental factors on the size and level of perfection that are attained by crystals of hydroxyapatite. If such correlations can be established, they will be used in attempts to assess the role various biological influences may have on the process of hydroxyapatite formation in hard tissue.

Major Findings:

This study is presently in the stage of data acquisition. No analyses have yet been undertaken.

Significance to Dental Research:

The wide variations in crystal size and perfection of bone and dentin hydroxyapatite reported in the literature suggest that a reevaluation of x-ray line broadening methods is in order. The importance of this reevaluation lies in the fact that x-ray line broadening analysis is potentially one of the most powerful techniques available in studying the crystals of biological apatite. Knowledge of such crystal parameters as size and perfection may be extremely useful in understanding the mechanism of crystallization of hydroxyapatite in hard tissue as these parameters are reflections of the conditions existing at the time of mineral formation.

Proposed Course of Project:

Once the validity of applying x-ray line broadening procedures to the analysis of the size-strain content of synthetic hydroxyapatite has been established, the procedures will be applied to the study of crystal size and strain in apatites of biological origin. Special emphasis will

be placed on correlating changes in size and strain with the biological age of the hard tissue and comparison of the time dependencies found for these changes with those found in synthetic systems aged under comparable physiological conditions.

Part B

Not included.

Serial No. NIDR-51 (63)
1. Histology & Pathology
2. Crystal Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

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-52

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 determine the structural details and composition of the inorganic phase of teeth and bones. 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. An understanding of the vibrational spectra of pure synthetic substituted apatites and related compounds is necessary in order to determine the structural details and composition of the inorganic phase(s) of hard tissue. Hence, this study entails assignment of the infrared absorption spectra of pure apatites, of related calcium phosphates, and of substituted apatites including hydroxy-, fluor-, chlor-, oxy-, and mixed apatites containing two or more of the above anions in various proportions. Structural details,

including hydrogen bonding, molecular orientation, molecular point groups, site symmetry, and non-equivalent structural sites for identical groups are inferred from the infrared spectra. Specialized infrared techniques involve utilizing reflectance, polarization, low and high temperature and high pressure devices in obtaining spectra. Methods are developed for synthesis and purification of the compounds studied spectroscopically which require design and construction of specialized apparatus to maintain experimental conditions, such as high temperature and pressure required to form the apatites in well-crystallized form. Isotopically substituted analogues are prepared to facilitate assignment of specific infrared absorption bands. The techniques are supplemented by chemical analyses to ascertain purity and chemical composition of the preparations.

Major Findings:

Samples of pure well-crystallized hydroxyapatite, Ca(OH)A, containing approximately 80% of the total oxygen replaced with O^{18} have been prepared.

Three reaction approaches were tried. Reaction I involves oxidation of elemental phosphorus by H_2O^{18} to PO_4^{18} at high temperature and reaction with CaO and H_2O^{18} to form apatite. Reaction II allows an O^{18} substitutional pathway in the PO_3 to PO_4 conversion by reacting $3\ Ca(PO_3)_2 + 7\ CaCO_3$ with excess H_2O^{18} to form the apatite, and Reaction III involves direct O^{18} exchange with the O^{16} atoms of the PO_4 groups by treating Ca(OH)A with H_2O^{18} at high temperature. Reaction II, found most suitable, was accomplished by igniting the solid mixture with excess H_2O^{18} (total O^{16} to O^{18}, 1 to 10) in a sealed Pt tube at 600°C for 2 days and then at 900°C for 5 days.

Spectra of the apatite containing approximately 80% of the O^{16} replaced with O^{18} showed distinct frequency shifts which establish and confirm the ν_3, ν_1 and ν_4 phosphate assignments. On the basis of isotopic substitution (O^{18}, H^2), band intensity, iso-structural analogues and temperature dependance, the weak Ca(OH)A band at 472 cm^{-1}, previously assigned by other investigators to a difference tone, is reassigned to the ν_2 phosphate mode.

The Ca(OH)A band at 342 cm^{-1}, previously assigned to ν_2, is reassigned to Ca - OH translational motion as based on O^{18} and H^2 spectral shifts, band intensity, temperature dependance and a decrease in intensity upon thermally removing OH groups. The Ca(OH)A band at 280 cm^{-1}, previously assigned to the ν_2, PO_4 mode, and the unreported band at 230 cm^{-1} are both assigned to Ca - PO_4 lattice modes.

The ν_3, ν_1, ν_4 and ν_2 phosphate fundamentals for strontium hydroxyapatite, Sr(OH)A, and barium hydroxyapatite, Ba(OH)A, have been assigned by analogy with those of Ca(OH)A, and the OH modes have been assigned as based on shifts on deuteration. Five of the Sr(OH)A bands assigned by Bhatnagar have been reassigned as based on this study.

At -185°C the corresponding vibrational modes of the Ca, Sr and Ba hydroxyapatites show systematic shifts and intensity changes. As an effect of lattice contraction on cooling, all the vibrational modes, except the OH librational modes, shift to higher frequency. The bands assigned to the external modes shift more on cooling (lattice contraction) than the internal modes which is expected and is consistent with the assignments.

Plots of the frequencies of the corresponding vibrational modes in the Ca, Sr and Ba hydroxyapatites against the increasing cation mass, ionic radius or a axis lattice dimensions of the apatites are almost linear. Except for the frequencies of the OH stretching modes which increase in the sequence, Ca<Sr<Ba, all the other plots decrease. The bands assigned to metal-phosphate lattice modes show greater decreases to lower frequencies than the internal phosphate modes which is also consistent with the assignments.

The OH vibrational modes are of primary interest in the Sr and Ba hydroxyapatite analogues. The Ba, Sr and Ca hydroxyapatite OH stretching modes occur at 3605, 3591 and 3572 cm^{-1}, respectively, and the OH librational modes at 420, 535 and 630 cm^{-1}, respectively. The decrease in the OH stretching frequency and the marked increase in the librational frequency in the Ba, Sr, Ca hydroxyapatite sequence are suggestive of a hydrogen-bond trend arising from decreasing O—H---O separations. The OH ions in Ca(OH)A, about 3.44 A apart, are located on, and oriented parallel to, the sixfold screw axis. Assuming equivalent OH positions in Sr(OH)A and Ba(OH)A, gives OH separations of 3.64 and 3.85 A, respectively. The maximum O — O separation for O— H---O bonding has been estimated to be about 3.3 A which minimizes the possibility of (O—H---O——H) bonding along the c axis in these apatites. The closest oxygen approach to the OH ion is from the three oxygens of the three phosphate groups which surround the OH ion. This OH to phosphate oxygen distance is 3.043 A (Young, et al.), and the corresponding estimated distances in the Sr and Ba hydroxyapatites are, assuming the same OH position, 3.14 and 3.28 A, respectively, all of which are close enough for weak hydrogen bonding.

At -185°C the OH stretching modes of these apatites shift about 2 cm^{-1} to higher frequency and the librational modes about 3 cm^{-1} to lower frequency. The directions of these shifts do not support OH---OPO_3 bonding, and additional studies will be necessary to establish if this type of bonding occurs in these apatites.

Assignment of the low-frequency modes in Ca(OH)A has afforded a means to assign the calcium fluorapatite, CaFA, and calcium hydroxy fluorapatite, Ca(OHF)A, bands in this region. The CaFA bands ascribed by other investigators to a $\nu_3 - \nu_4$ difference tone (472 cm^{-1}) and the bands at 325 and 280 cm^{-1} to the ν_2 phosphate mode have been reassigned according to this study as follows: 472 cm^{-1}, ν_2, PO_4; 325 cm^{-1}, Ca-F translation and 280 cm^{-1} to a Ca - PO_4 lattice mode. The OH stretching and librational modes in Ca(OHF)A have previously been assigned and from the number and shifts in these bands two different O—H---F interactions were indicated which has been confirmed by combined NMR and x-ray studies by other investigators. The Ca - OH translational mode in Ca(OH)A has been assigned and occurs at 342 cm^{-1}. This band shifts to higher frequency as a function of increasing fluoride content which is, also, consistent with O—H--F bonding.

Significance to Dental Research:

Characterization and assignment of the infrared absorption bands of pure apatites containing biologically relevant anions and those of related calcium phosphates are essential in establishing the structural details and composition of the inorganic phase(s) of teeth and bones. The type(s) and degree of incorporation of hydroxyl, fluoride, chloride and carbonate ions, water, acidic phosphate and different cations in biological apatites have bearing on the chemical and physical properties of hard tissue. Identification of these ultrastructural details is fundamental in inderstanding the structural chemistry of hard tissue and, more significantly, these details offer avenues to affect the building and remodeling of hard tissue and, in turn, its characteristics.

Some of the postulated reactions of fluoride with enamel, such as, replacement of hydroxyl and carbonate ions, have not been clearly established by physical or chemical methods. 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). Spectral assignment and interpretation of the infrared absorption spectra of synthetic $Ca_{10}(PO_4)_6$ $(OH_{2-x}F_x)$, where x has been varied in small increments from 0 to 2, have revealed structural details which include O-H---F bonding and preferential O-H---F interactions rather than separate aggregation of the OH and F ions. The marked decrease in solubility, relative to hydroxyapatite, of $Ca_{10}(PO_4)_6$ $(OH_{2-x}F_x)$ containing small amounts of F reported by Rathje, most probably arises from the enhanced chemical stability resulting from hydrogen bonding. Preliminary spectral studies show O-H---F bonding in enamel containing a few tenths weight % of F.

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.

Proposed Course of Project:

Completion of low-temperature studies of calcium hydroxyfluorapatite and three manuscripts describing these studies.

Continuation of the chemical and infrared studies of carbonate containing apatite.

Part B:

Publications:

A summary of part of this work was presented at the International Symposium on Structural Properties of Hydroxyapatite and Related Compounds held at the National Bureau of Standards, Gaithersburg, Maryland, on September 12-14, 1968, and the summary is to be included in a book covering the symposium.

Serial No. NIDR-52
1. Histology and Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Odontopathic Bacterial Plaques: Etiologic Factors Pathological Sequelae, Therapeutic Measures

Previous Serial Number: NIDR-53

Principal Investigator: Dr. P. H. Keyes

Other Investigators: Dr. R. J. Fitzgerald, Dr. H. V. Jordan, Dr. P. N. Baer, Dr. M. A. Hicks, Dr. H. Swerdlow Dental Services Branch, NIDR

Cooperating Units: Dr. S. S. Socransky, Forsyth Dental Center, Boston, Mass.; Dr. Harry H. Eisenberg and Mr. Stephen Bellock, Lincoln State School, Lincoln, Ill.

Man Years:

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

Project Description:

Objectives:

1. To gain insight into the various factors conducive to colonization of the teeth by pathogenic bacteria. 2. To explore the potential of various therapeutic measures to control colonization itself and/or the pathogenic effects associated with bacterial plaque infections which follow colonization.

Methods Employed:

Animal model systems use hamsters and rats to assess the pathogenic interactions between diets and bacteria. Routine bacterial sampling of dental plaque samples are used. Human volunteers have participated in mouth wash studies.

Experiments and Major Findings:

1. Studies in collaboration with Dr. P. N. Baer revealed that 3 strains of bacteria (diphtheroidal rods) isolated by Dr. Socransky from rats prone to develop periodontal lesions will colonize the cervico-radicular surfaces of non-infected hamsters and OM rats fed appropriate diets. The colonization and subsequent plaque formation are conducive to gingival distortion, periodontal detachment, pocket formation, alveoloclasia, and calculus formation.

2. Additional tests with bacterial isolates supplied by Dr. Harold V. Jordan were undertaken in hamsters and rats. Isolates from the rat were pathogenic and induced lesions similar to those described above. Isolates of human origin, on the other hand, were not conducive to a disease syndrome which could be replicated in repeated trials. Several variations in diet and methods of inoculation were assessed, but methods for satisfactory induction of disease which human isolates remain elusive.

3. In collaboration with Dr. M. Hicks (Dental Services Branch, NIDR) preliminary tests in human volunteers disclosed that a mouthwash containing 10,000 units of dextranase/cc will partially disperse bacterial plaques accumulated over a 72 hour period during which the subjects rinsed with sucrose solutions and discontinued oral hygiene measures. The subjects tested in this study had been identified previously as carriers of dextranogenic caries-conducive streptococci.

4. A contract (PH 43-68-1245) with the Lincoln State School, Lincoln, Illinois, was arranged for the purpose of studying the oral health status of mongoloid patients from infancy to adulthood. Prior examination of groups of mongols suggested that these patients suffered from progressive dental plaque infections which commenced in infancy, advanced with unusual rapidity, and soon caused major periodontal pathosis and loss of teeth. The hypothesis under exploration is that most mongols are prone to experience severe periodontal pathosis as a consequence of cervico radicular plaque infections and their apparent sub-normal resistance to disease. Clinical findings are recorded and bacterial smears as well as samples of plaque are obtained in order to determine if meaningful correlations can be identified. Dr. Harry Eisenberg, Mr. Stephen Bellock and co-workers have been conducting the studies at Lincoln State.

5. Teeth scheduled for extraction from older patients were treated with an experimental occlusal sealant (isobutyl 2-cyanoacrylate and alumina). After extraction the teeth were examined grossly and cut into sections. Although the filling material is quite soft, it remained in place for the rather short experimental periods used. The occlusal surfaces were pretreated with an acid to improve adhesion but the crevices were not cleared of foreign matter, thus preventing an evaluation of the potential of this material as a sealant.

Significance to Dental Research

The concepts developed over the past few years are of high significance not only to dental research but also to the understanding and treatment of the major disease problem in dental medicine.

It is now apparent that the microbial colonization of tooth surfaces by certain bacterial pathogens can lead to extraordinarily invasive diseases. The lesions produced have been recognized for centuries and are now identified by such nondescript terms as "dental caries" and "periodontal disease." Zoogleic plaque infections cause the cavitation of the crowns and roots of teeth as well as the destructive inflammatory reactions in the periodontal attachment tissues. The autogenous exfoliation of teeth may well be the normal defen e of the host against serious pathogens which have attacked the cementum, attached themselves thereto, and progressively invaded the periodontal space.

If specific plaque forming pathogens can be implicated as clearly in dental plaque infections in humans as they have been in experimental animals, steps can be undertaken to develop target drugs and measures of therapy.

If the postulations concerning the etiology of periodontal pathosis in mongols be correct, it will clarify important aspects of the much misunderstood oral disease in this group. The disclosure of plaque infection as an essential etiologic component would open the way for more effective treatments not only in mongoloid patients but also in all handicapped persons. Numerous chemotherapeutic measures can be used.

The finding that dextranase partially disperses dextranous plaque on human teeth, supports the concept that dextrans may contribute to colonization and plaque formation.

Proposed Course of Project:

Studies will continue to assess the odontopathic potential of bacteria isolated from human teeth extracted because of radicular plaque disease. Different combinations of diet, bacteria, and methods of inoculation will be used.

The contract with the Lincoln State School will be continued. Investigators there will eventually assess the potential of various drugs to cure and control plaque infections. Both dental and medical personnel are eager to begin clinical trials with various agents and methods of administration. The impressions gained from preliminary studies along these lines have been described as "remarkable." With the therapeutic responsibility in the hands of the clinicians at the school, it should be possible to obtain meaningful data in regard to

the treatment of infectious plaque deposits, microbiological shifts in the oral flora, clinical responses of the oral tissues, and complicating reactions.

Additional studies with dextranase will be undertaken to determine its potential effect on plaque formation. More effective methods of delivery will be explored.

Part B:

Publications

1. Englander, H. R., and Keyes, P. H.: Acid production in the dental plaque of hamsters protected from dental caries with sodium fluoride. J. Oral Ther. and Pharm. 4:382-287, 1968.

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 dental calculus. J. Periodont. 39:86-88, 1968.

3. Larson, R. H., Keyes, P. H., and Goss, B. J.: Development of caries in Hunt-Hopper caries-susceptible and caries-resistant rats under different conditions. J. Dent Res. 47:704-709, 1968.

4. Keyes, P. H. A discussion of present and future measures for dental caries control. J. A. D. A. - accepted for publication.

5. Keyes, P. H. Zoogleic plaque infections conducive to dental caries. Proceedings - Pan Pacific Congress of Dental Research. Tokyo, Japan. April 1969 - accepted for publication.

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

During the past year, the administrative organization of the laboratory has remained essentially unchanged. The five sections within the laboratory all have well established and productive programs. The only major change has been the return to laboratory research of the Chief of the Pharmacology Section who was assigned to South America during 1966-68.

During FY 1968 the laboratory has become somewhat reduced in size (from a total of 51 to 46 persons, including guest workers) reflecting the general reduction in positions and funds. This has resulted in some hardship and loss of productivity but the effect has been smaller than was feared. There has been some compensation in the resulting partial alleviation of space problems but this is still a major source of inconvenience.

As in the past, the programs of the laboratory have used the services of approximately equal numbers of permanent senior staff and postdoctoral personnel here under one of the several NIH programs, or as guest workers. Training, therefore, is an important byproduct of the program as well as a source of scientific talent. With a shortage of positions and funds, there is a trend away from postdoctoral personnel which may later have a large effect on the character of the laboratory.

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

Protein Chemistry Section

The chemistry of the collagen molecule continues to be the major project. Beginning a number of years ago with the isolation of the α chains of collagen, a systematic attack has been made on the chemistry of the collagen molecule. The polypeptides obtained by CNBr cleavage of both $\alpha 1$ and $\alpha 2$ chains of rat skin and chick bone collagen have now all been isolated and characterized by molecular weight and amino acid composition. Most of the smaller peptides have been sequenced and the carbohydrate has been located in the $\alpha 1$ chain of rat skin collagen. Work done here and in other laboratories (by investigators trained here) has now accounted for more than 10% of the amino acid sequence of the $\alpha 1$ chain. It is now possible to begin to correlate sequence with other aspects of structure and with function.

Comparative studies of collagen from different species and from different tissues of one species have been a major aim of the program. Rat skin collagen can now be compared in some detail with rat tail tendon collagen and chick bone and skin collagens are also well characterized. It appears that the $\alpha 1$ and $\alpha 2$ chains of the two rat collagens have identical amino acid sequences although modifications are introduced after assembly of the polypeptides chains that may be important to structure and function. The same conclusion applies to chick skin and bone collagen. When the two species are compared, there are differences in sequence of the type seen in other proteins. However, the collagens are very similar and clearly homologous.

Although skin and tendon, in the rat, and skin and bone, in the chick have the same basic primary structure, the conclusion that all collagens in a species are derived from the same structural genes does not necessarily follow. In fact, current studies show that cartilage collagen from the chick does not have the same primary structure as skin and bone collagen. As in the case of interspecies comparisons, the chains are clearly homologous, but differ in such a way that they must have arisen from different genes. The cartilage collagen is also unusual in that it appears to have only one kind of α chain which corresponds to $\alpha 1$. It appears that more than one set of structural genes for collagen synthesis is available to an animal but that in some cases the same set is utilized in tissues of quite different character.

To obtain further comparative data, studies have begun to characterize collagen from a primate. A contract has been let to make several baboons lathyritic so that they will provide a large yield of extractable collagen. These studies are preliminary to detailed studies on human collagen which, however, is difficult to obtain in sufficient quantity.

The cross-linking of collagen is critical to function. The location and mode of intramolecular cross-linking has been shown in earlier studies (see Connective Tissue Section). Current studies are on intermolecular cross-links which predominate in many tissues such as bone. Evidence has been obtained which suggests a cross-linking site near the C-terminal end of the collagen molecule in addition to the one at the N-terminal end previously characterized. Both sites appear to be involved in intermolecular cross-linking.

Chemical studies on cross-linking of collagen have been supplemented with <u>in vivo</u> studies done under contract in Israel. It has been shown that isolated and purified collagen placed in a diffusion chamber and returned to the animal undergoes normal maturation and cross-linking. The technique allows the study of various modified forms of collagen as well as the effect of the state of the animal on the maturation process.

A serious limitation to the study of collagen is the insolubility of most collagen. This problem is being solved by cleaving insoluble collagen with CNBr. If the products obtained from a soluble form of the collagen have been characterized, many of the products obtained from an insoluble collagen can be readily identified and any new products arising from cross-linking or other changes, possibly including pathological changes, can be recognized.

Turnover of collagen, as in wound healing and bone remodeling, requires a specific enzyme. Studies on the collagenase from leucocytes have been directed toward the purification and characterization of the enzyme. The enzyme has a molecular weight of about 64,000 and a K_m for native collagen of about 6 μM. It is inhibited by EDTA and certain metals. It requires Mg^{++} or Mn^{++} and has the unusual property that it is inhibited by thiols and activated by agents which react with thiols.

An understanding of the way in which the chains of collagen form a triple-stranded helix is important not only to an understanding of collagen stability but also to the more general topic of chain-chain interactions in protein structure. A particularly simple system is available in the small peptides

from CNBr cleavage of collagen. Helix formation in one of these, α1-CB2, having 36 amino acids and a known sequence, has been studied by optical rotation. The rate constants for helix formation and melting and the thermodynamic parameters describing the equilibrium state have been determined. The formation of helix is third order, consistent with the requirements for the assembly of three chains, while helix melting is first order. With this relatively small peptide the rate limiting step is the initial aggregation and nucleation.

Collagen fibers provide a model system for the study of the interconversion of mechanical and chemical energy. The shrinkage of fibers is a function of temperature, the force on the fibers, and the presence of stabilizing or destabilizing agents in the environment. These interrelations have been examined theoretically and experimentally. A model has been devised for the mathematical treatment of experimental data and the thermodynamic parameters describing the system have been obtained.

In addition to studies on collagen, two new projects have been initiated this year -- the characterization of the progesterone receptor in chick oviduct and the chemistry of muscle proteins. In both cases potentially significant preliminary results have been obtained which will be pursued.

Connective Tissue Section

The program of this Section complements the collagen studies in the Protein Chemistry Section. The major project has been to purify and characterize the enzyme(s) responsible for cross-linking of collagen and elastin. The first step is the conversion of certain lysyl side chains in peptide linkage to an aldehyde, allysine. The enzyme, designated lysyl oxidase, was first detected in studies done here and has now been further characterized. It has a molecular weight of about 70,000 and is copper dependent; it is inbibited by lathyrogens such as β-aminopropionitrile. It has been shown to act on both elastin and collagen. In the latter case it has been shown that the same lysyl residue that is the substrate *in vivo* is converted *in vitro* to allysine.

Once the aldehyde is formed, cross-linking can proceed spontaneously perhaps by several pathways. This has been found to occur in purified, reconstituted collagen fibrils incubated *in vitro*. One of the products is the intramolecular aldol cross-link derived from two molecules of allysine previously found *in vivo*.

Cell culture systems are being utilized to study collagen biosynthesis. Of particular interest is the control mechanism that normally produces two α1 chains and one α2 chain to make collagen molecules. In culture, the media contains excess α2 chain suggesting that either the control is lacking under culture conditions or that wastage of α2 is normal.

Knowledge derived from the basic studies on collagen chemistry is being utilized to look for "molecular" defects in hereditary diseases involving connective tissue. Cells from Marfan's or Ehlers-Danlos patients grown in culture secrete a larger proportion of the collagen they make into the medium that do normal cells, suggesting that there is a defect in the ability to form collagen fibrils This observation may correlate with the fragility of connective tissues in these disorders.

Enzyme Chemistry Section

Studies carried out over the past several years have been primarily focused on the basic mechanisms by which enzymes function as organic catalysts. An enzyme, guinea pig liver transglutaminase, has been chosen for intensive study because it displays catalytic properties that are characteristic of a number of classes of enzymes, and because it, or a similar enzyme, has the important physiological property of stabilizing fibrin clots by the introduction of cross-links. In an in vitro system using model substrates, the order of addition of substrates and release of products has been formulated. The roles of the essential divalent cation, Ca^{++}, in substrate binding and in the transfer of an acyl group from a stable intermediate acyl enzyme to an acceptor amine have been partially elucidated. A functional group of pK 6.5 has been implicated in the binding of Ca^{++}. Specific chemical modifications of the enzyme protein, resulting in differential catalytic changes, have given evidence that sulfhydryl groups are involved in substrate binding as well as in catalysis. The stereochemical orientation of amino acid side chains in the enzyme active site is evident from studies with D- and L- isomers of substrates, inhibitors and activators.

Cell Biology Section

A number of aspects of growth regulation in lymphocytes and related cell types are currently under investigation. Detailed studies of the alterations in RNA metabolism associated with the shift of lymphocytes from the resting to the resting to the growing state have been carried out. Control of ribosome production has been identified as a major regulatory area in these cells. At least three sites of control have been studied, (a) the synthesis of ribosomal RNA precursor, (b) the rate of conversion of rRNA precursor, to subsequent forms of ribosomal RNA, and (c) the survival and utilization of newly synthesized rRNA molecules. Each of these sites appears to have its own control mechanism which is independently altered upon growth stimulation. Further knowledge concerning these control mechanisms is currently being sought, since they must constitute an important means of cell growth regulation. This will be of considerable importance in furthering our understanding of neoplasia, cell and tissue growth and differentiation.

The nature of the interaction of lymphocytes with other cell types and with antigenic substances which induce lymphocyte growth is under study. Comparison of the response of cells from normal subjects and patients with a variety of diseases involving the lymphoid-immunological system has helped to clarify the nature of the immunological abnormality in these patients. Thus, patients with aphthous stomatitis have been shown to be hyporesponsive to antigens from microorganisms widely thought to have an etiological relationship to their disease. The same was found to be true of patients with rheumatic heart disease.

The nature of the cooperation between macrophages and lymphocytes in responding to an antigenic challenge is being studied. It has been found that macrophages are effective in enhancing the growth response of lymphocytes to antigenic challenge when the lymphocytes are derived from sensitized animals. The macrophages need not have had prior contact with antigen, however, indicating that the immunological speificity of this cell-cell interaction is determined

by the lymphocyte. In other studies, the ability of an antigen to produce a response was shown to depend on the quantitative relationship between antigen and antibody in the experimental system. Thus, complexes between antigens and small amounts of specific antibody greatly enhance lymphocyte response to the antigen, while the presence of excess antibody inhibits the reaction. A number of factors therefore contribute to the ability of lymphocytes to grow in response to a given antigenic challenge.

All of the above investigations have considerable relevance to the important area of delayed hypersensitivity, including the problem of graft rejection and acceptance, since it is the recognition and response to the foreign proteins or tissues on the part of the lymphocyte-macrophage system which initiates the graft rejection phenomenon.

A sensitive binding assay has been developed to study the interaction of phytohemagglutinin, a non-specific lymphocyte growth stimulant, with the cell. Using this technique it has been found that binding of the growth stimulant to the cell surface is an initial step which is followed by a second independent, reaction producing cell growth induction.

In another area related to growth in animal cell systems analysis of the forms of RNA produced during replication of an RNA virus were performed, using new developed chromatographic techniques. These techniques also promise to provide an improved approach to the study of RNA metabolism in non-infected cells. A new class of actinomycin resistant RNA synthesis has been detected and the RNA produced has been partially characterized using the new chromatographic methods. The occurrence of this class of RNA in a variety of cell types indicates the existence of an RNA synthetic activity not previously described in animal cells. Because of their extreme sensitivity, the techniques developed in this area are also being applied to the problem of detection of RNA viruses in malignant cell lines which may play a causal role in neoplasia.

Pharmacology Section

The current program is directed largely to studies of the normal development of the oral-facial region and the role of exogenous and endogenous factors. Of particular interest is the development of cleft palate and other malformations.

The use of experimental animals in these experiments is of great importance. Timed studies of normal organogenesis of the oral region of the Rhesus monkey have shown that palatal development takes place during days 42-50 of gestation with closure occurring during day 46. This information now makes available for the first time a subhuman primate for the investigation of cleft palate.

Two groups of compounds, benzhydrylpiperazines and lathyrogens such as β-aminopropionitrile and semicarbozide, produce cleft palate in a variety of experimental animals. The former group of compounds is effective in rats when administered on days 13 and 14 of gestation while the latter is effective on day 15. This observation, together with the appearance of a different type of pathology, provides a valuable tool for the study of the etiology of cleft palate. The lathyrogens are of particular interest since it is known that they inhibit cross-linking of collagen. Collagen may play a critical struc-

tural role at the time of palatal closure.

It is well known that there are strain differences in the response to certain teratogens. In the case of 6-aminonicotinamide, liver studies have demonstrated that the difference resides in the mitochondria. Further characterization is required, but this appears to be the first mitochondrial mutant identified in mammals.

The complex events that may occur when a pharmacological agent is administered systemically often obscure the effects at a specific anatomical site. To avoid this problem, a technique has been developed to apply a teratogenic agent directly to a specific area of a developing fetus. Using this technique, the mechanism of action of the teratogen norchlorcyclizine has been approached by examining the ability of structural analogs to bind to bovine nasal septum and displace Ca^{++}. A correlation was found between the ability to displase Ca^{++} and the teratogenic activity.

Serial No. NIDR-53 (52)
1. Biochemistry
2. Protein Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Analytical and Structural Studies on Collagen

Previous Serial Number: NIDR-19

Principal Investigator: Dr. K. A. Piez

Other Investigators: Dr. P. P. Fietzek, Dr. J. Vuust, Dr. J. R. Daniels, Dr. M. M. Rubin

Cooperating Units: Dr. A. H. Kang, Massachusetts General Hospital, Boston, Massachusetts, Dr. A. Katchalsky, Weizmann Institute, Rehovot, Israel

Man Years:

Total:	6 1/2
Professional:	4 1/2
Other:	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) to characterize the α chains of various collagens, (2) to examine the interaction of collagen and the enzyme, collagenase, which has a specific role in collagen degradation, (3) to study formation of the collagen helix by utilizing a small peptide from collagen, and (4) to study the interaction of alyotropic salt, LiBr, with collagen and its dependence on force.

Methods Employed:

Usual laboratory methods.

Major Findings:

(1) The characterization of collagen α chains by specific cleavage with CNBr has been applied to several collagens. In current studies the α2 chain of rat skin collagen has been cleaved into six polypeptides ranging in size from a tripeptide to ones having molecular

weights of about 30,000. Together the peptides account for all of the weight and amino acids in the α2 chain. This completes the characterization of all the CNBr peptides from rat skin collagen and opens the way to more detailed studies and the elucidadation of the complete structure of a collagen molecule.

To provide comparative data, collagens other than rat skin collagen are also being studied. In collaborative studies with Dr. A. Kang at Massachusetts General Hospital, the CNBr peptides from the α1 and α2 chains of chick skin collagen have been isolated and characterized. It has been shown that these peptides are similar and probably closely homologous to the peptides from rat skin collagen. It has also been shown that the NH_2-terminal cross-link region of the two collagens are very similar.[2] In both, a specific lysyl residue is converted to an aldehyde which is the precursor of cross-links.

(2) Since collagen must be degraded during growth (remodeling of bone, for example, and during wound healing, there must be a control mechanism and one or more enzymes that bring about degradation. A vertebrate collagenase from leucocytes that has a very selective mode of attack on native collagen is being studied to elucidate the control mechanism. The collagenase has been partially purified and is free of nonspecific proteolytic activity. The enzyme has the following properties: molecular weight about 64,000, maximal activity at pH 7-8, a large temperature dependence, and a K_m for native collagen of about 6 μM. The specificity of the enzyme is shown by the observation that although it cleaves denatured collagen, it has a greater affinity for the native protein. However, the specificity resides in part in the amino acid sequence at the site of cleavage as shown by the observation that denatured collagen is cleaved predominantly at the same place as native collagen. Enzymatic activity is inhibited by Zn^{++}, thiols and EDTA. The latter can be reversed by addition of Mg^{++} or Mn^{++} but not of Ca^{++}. Agents which react with thiols increase activity as much as 10-fold.

(3) The collagen molecule contains three helical chains wrapped into a larger triple-chain helix. Helix formation and melting is being studies with a peptide of 36 amino acids and known sequence from the α1 chain of rat skin collagen. At room temperature the peptide is an unstructured monomer in solution. When cooled a triple-chain collagen-like molecule is formed. Unlike similar studies on larger polypeptide chains, the process is fully reversible. Equilibrium studies show that ΔH for the reaction is about -86 kcal/mole. Kinetic studies are consistent with a third order reaction for helix formation and a first order reaction for melting as predicted for a collagen-like molecule. The rate of helix formation has only a small negative dependence on temperature while the rate of helix melting has a large positive dependence on temperature.

(4) Ther reversible melting of a collagen fiber (shrinkage) in aqueous environment is akin to the process described above for a

small peptide. However, there is the added parameter of force since the fiber exerts a measurable tension during melting. The presence of certain salts such as LiBr lowers the skrinkage temperature. In collaborative studies with Dr. A. Katchalsky, Weizmann Institute, Rehovot, Israel, the thermodynamics of the relationship of force to temperature and concentration of LiBr has been examined theoretically. The prediction is made that LiBr brings about melting by preferential binding to the denatured state and that the application of force to the melted fiber will renature the collagen and cause the release of excess bound LiBr. Experimental studies utilizing cross-linked collagen fibers suspended under varying forces in LiBr solutions have provided data consistant with the theoretical model. A two-state allosteric model adequately describes the process and gives information concerning the nature of the melting process.

Significance to Dental Research

Collagen is the major structural protein of connective tissue including skin, bone, and teeth. An understanding of its chemistry is fundamental to an understanding of connective tissue function in the normal animal and in disease states. The specific objectives of this project have potential significance in the following ways. (1) A complete characterization of the α chains of collagen will permit the comparison of collagens from normal and disease states and the location of "molecular" defects such as occur in hereditary diseases. A possible critical place where defects may arise is in the cross-linking of collagen which is necessary to produce a functioning tissue. (2) Since the degradation of collagen is not only a normal process necessary for growth and repair, but occurs as part of many diseases a study of the process may lead to a rational approach to the cure of diseases where collagen is degraded. These include periodental disease and arthritis among others. (3) The stability of collagen, and therefore its proper functioning, is dependent on its helical structure. Minor alterations in amino acid sequence, as may occur in hereditary disorders, or alterations in other chemical characteristics of collagen may affect helical structur and thereby function. (4) The relationship between force, the conformational state of the components in an organized structure, and the environment is utilized by all living things to convert chemical to mechanical energy. Muscle action is one of the most obvious examples of a mechanochemical process. The collagen fiber provides a particularly simple mechanochemical model which is amenable to experimental and theoretical analysis.

Proposed Course of Project:

Two phases of the project have been completed. There are the studies on collagenase and on collagen fibers as a mechanochemical system. The characterization of collagen will continue along the lines already established, although some emphasis will shift to other projects that have developed in parallel or are offshoots of this one.

Publications:

Piez, K. A.: Cross-linking of collagen and elastin. Ann. Rev. Biochem. 37, 547-570, 1968.

Piez, K. A.: Molecular weight determination of random coil peptides from collagen by molecular sieve chromatography. Anal. Biochem. 26, 305-312, 1968.

Piez, K. A., Bladen, H. A., Lane, J. M., Miller, E. J., Bornstein, P., Butler, W. T., and Kang, A. H.: Comparative studies on the chemistry of collagen utilizing cyanogen bromide cleavage. Brookhaven Symposia in Biology 21, 345-357, 1969.

Kang, A. H., Piez, K. A., and Gross, J.: Characterization of the cyanogen bromide peptides from the $\alpha 1$ chain of chick skin collagen. Biochemistry, in press.

Fietzek, P. and Piez, K. A.: Isolation and characterization of the cyanogen bromide peptides from the $\alpha 2$ chain of rat skin collagen. Biochemistry, in press.

Rubin, M. M., Piez, K. A. and Katchalsky, A.: Equilibrium mechanochemistry of collagen fibers. Biochemistry, in press.

Serial No. NIDR-54 (66)
1. Biochemistry
2. Protein Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

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

Previous Serial Number: NIDR-20

Principal Investigator: Dr. K. A. Piez

Cooperating Units: None

Man Years: None, under contract

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, utilizing PL 480 funds under Research Agreement #645141 in the amount of 249,741 Israeli Pounds (about $75,000) for a three year period ending December 15, 1968. The project has employed half the time of Dr. Shoshan and 2 man years of technical personnel for each year of the agreement.

The studies utilize diffusion chambers that can be implanted in animals and later removed for detailed study. Collagen of various types and after various pretreatments is placed in the chambers and the chambers are placed under the skin of guinea pigs. The collagen is later removed and re-examined for changes in cross-linking by quantitative determination of the content of cross-linked components and by the extractibility of the collagen as a measure of late stages of cross-linking.

Major Findings:

Recently synthesized collagen, as yet uncrosslinked, when placed in diffusion chambers, undergoes cross-linking, showing that the normal process continues under the experimental conditions. A significant decrease in cross-linking could be obtained by pretreating the native collagen with enzymes which remove a portion of the molecule critical to cross-linking or by treating the recipient animal with a lathyrogen such as β-aminopropionitrile. This compound is known to inhibit the enzymatic production of aldehydes which is a necessary first step to cross-linking. These results were consistent with other studies on the chemistry and biosynthesis of cross-links and provide a direct demonstration of the lysine-to-aldehyde-to-cross-link conversion that is an important part of the maturation of collagen.

On the basis of the chemical and histological findings of these studies, experiments were devised to study the effect on wound healing of reconstituted collagen preparations. In preliminary experiments, collagen solutions applied to incisions in the skin or to bone fractures in experimental animals promoted healing. Histological examination showed less hemorrhage and a more rapid cell infiltration.

Significance to Dental Research:

The maturation of collagen is a fundamental process necessary for the proper function of connective tissue. These implant studies permit a careful control and evaluation of experimental conditions and will lead to a better understanding of the process. 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 initial objectives of the study have been largely satisfied and the contract period has ended. It is hoped to obtain a one-year extension of the contract to study some observations that were unexpected and may lead to a more complete understanding of cross-linking.

Part B: Not included.

Serial No. NIDR-55-(62)
1. Biochemistry
2. Protein Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: The Chemistry of Bone and Cartilage Collagens

Previous Serial Number : NIDR-21

Principal Investigator: Dr. E. J. Miller

Other Investigators: Dr. J. M. Lane, Dr. V. J. Matukus

Cooperating Units: Laboratory of Experimental Pathology, NIAMD, Dr. L. Sokoloff, Dr. E. H. Epstein, assigned to NIDR from NCI

Man Years:

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

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. Primate Collagen - In order to extend our studies to the collagen in tissues from animals close to the human on the evolutionary scale, a contract has been established with the Southwest Foundation for Research and Education for the raising and maintenance of three lathyritic baboons. It is hoped that successful induction of a state of lathyrism in an infra-human primate will enable us to obtain sufficient quantities of soluble collagen from a variety of tissues for biochemical characterization. The dollar value of the contract is $2291 and approximately 0.1 man years of the contractor's personnel are required.

Major Findings:

Bone collagen - Studies on the primary structure of the collagen chains in a calcified tissue (chick bone) have been continued utilizing acetic acid-soluble collagen from the femora and tibiae of lathyritic chicks. Cleavage of the $\alpha 2$ chain (containing five methionyl residues) at methionyl residues with cyanogen bromide (CNBr) gives rise to six unique peptides. Isolation and purification of the peptides was achieved by ion-exchange and molecular sieve chromatography. It was further shown that the solated peptides were present in equivalent amounts and that they accounted for all of the amino acids and all of the molecular weight of the $\alpha 2$ chain. In combination with our previous results on the two $\alpha 1$ chains of chick bone collagen, the study on the $\alpha 2$ chain completes the isolation and characterization of all the CNBr peptides derived from the collagen molecule of a calcified tissue. These peptides represent convenient starting materials for further studies on the primary structure of the protein and sequence studies have been initiated on the peptides which appear to be involved in intermolecular cross-linking of the bone collagen (see below). The methionyl residues of the $\alpha 2$ chain are distributed along the chain in such a manner that three of the peptides ($\alpha 2$-CB3, $\alpha 2$-CB4, and $\alpha 2$-CB5) are of approximately equal size and together they account for 95% of the amino acids and molecular weight of the chain. The remaining peptides ($\alpha 2$-CB0, $\alpha 2$-CB1, and $\alpha 2$-CB2) are relatively small. $\alpha 2$-CB0 is a tripeptide (sequence: gly-leu-met) and $\alpha 2$-CB1 and $\alpha 2$-CB2 contain 15 and 30 amino acids respectively. The CNBr peptides derived from chick bone collagen $\alpha 2$ have also been shown to be homologous to those derived from rat skin collagen $\alpha 2$. Of these peptides, $\alpha 2$-CB1 is of considerable interest. Similar to its homologue in rat skin collagen, $\alpha 2$-CB1 of chick bone collagen is derived from a nonhelical portion of the chain since it contains only two glycyl residues. Likewise, the two peptides each contain one lysyl residue although this residue is approximately 50% hydroxylated in the peptide from chick bone collagen. Digestion of chick bone collagen $\alpha 2$-CB1 with trypsin and isolation of the resulting peptides indicates that lysine and hydroxylysine occupy identical positions in the peptide (amino acid number 6 from the NH_2-terminus). Our previous studies on the CNBr peptides from the $\alpha 1$ chain of chick bone collagen have shown that $\alpha 1$-CB1 is similarly related to its homologue from rat skin collagen $\alpha 1$. Since the lysyl residues in rat skin collagen $\alpha 1$-CB1 and $\alpha 2$-CB1 have been shown to participate in the formation of intramolecular cross-links between the polypeptide chains, partial hydroxylation of these residues in bone collagen may play a significant role in the manner in which cross-linking occurs in the latter tissue. It is of interest that although the CNBr peptides from the collagen in

several tissues (rat skin, rat tendon, chick skin, and chick bone) have been examined, partial hydroxylation of the lysyl residue in α1-CB1 and α2-CB1 occur only in the bone collagen.

Since acid-soluble collagen from the bones of lathyritic animals contains only a small proportion of cross-linked components, and since it is virtually impossible to extract collagen from the bones of control animals without degrading the protein, a means was sought whereby the highly cross-linked collagen in the bones of control animals might be solubilized in order that an investigation of cross-linking might be performed. It was surmised that our previous work with soluble bone collagen had provided complete knowledge of the bone collagen molecule in terms of the CNBr peptides. Therefore, if cleavage at methionyl residues of insoluble bone collagen were successful, the complete mass of collagen could be brought into solution as recognizable peptides. Separation of the peptides obtained in this fashion would indicate which peptides were involved in cross-linking and eventually the chemical nature of the cross-link(s) could be determined provided the crosslinks were stable under the conditions employed for solubilization and chromatography of the peptides. It was found that all of the collagen in normal chick bones could be solubilized as CNBr peptides when cleavage was performed in 70% formic acid instead of 0.1 N hydrochloric acid as utilized for soluble collagens. Analyses of the material solubilized in the formic acid medium indicated that 90-95% conversion of methionyl residues to homoserine was achieved when the reaction was performed at room temperature for 4 hours with a 100-fold excess of CNBr (relative to methionine). These conditions were therefore utilized to cleave the collagen chains in three different collagen fractions which on the basis of their solubility characteristics and extracted components gave evidence of containing varying degrees of cross-linking. The three fractions were as follows: acetic acid-soluble collagen from the bones of lathyritic animals; acetic acid-insoluble collagen from the bones of lathyritic animals which can be solubilized only in solvents in which the protein is denatured (5M guanidine hydrochloride); and collagen from the bones of control animals which is totally insoluble in acetic acid and only sparingly soluble in 5M guanidine hydrochloride. Phosphocellulose chromatography of the first fraction after CNBr cleavage indicated that all of the peptides usually resolved on phosphocellulose, α1-CBO, α1-CB1, α1-CB,, α1-CB3, α1-CB6A, α2-CBO, α2-CB1,and α2-CB2 were recovered in anticipated amounts. Moreover, the peptides were qualitatively identical to those isolated after CNBr cleavage of isolated chains in 0.1 N hydrochloric acid indicating that cleavage at methionyl residues in 70% formic acid was not accompanied by unusually large amounts of non-specific cleavage. Agarose molecular sieve chromatography and carboxymethyl cellulose chromatography of

the more basic peptides not eluted as discrete peaks from phosphocellulose indicated similar results with regard to both the quantity and quality of these peptides, α1-CB7, α1-CB8, α1-CB6B, α1-CB4, α1-CB5, α2-CB3, α2-CB4, and α2-CB5. When identical chromatographic procedures were employed for the peptides from the two insoluble collagen fractions, it was readily observed that significant amounts of three peptides, α1-CB1, α2-CB1, and α1-CB6B were unaccounted for and presumably associated through cross-linking with other peptides. The view that cross-linking is responsible for the lack of stoichiometry in these peptides is substantiated by the observation that the apparent loss of each of the three peptides could be correlated with the solubility characteristics of the collagen fraction under investigation. For instance, 50% of the anticipated amount of α1-CB1 was unaccounted for in the acid-insoluble fraction of bones from lathyritic animals whereas approximately 90% of the peptide was unaccounted for in the acid-insoluble fraction of bones from control animals. These results also indicate that the cross-links in bone collagen remain intact under the conditions utilized to obtain the peptides. The peptide(s) to which each of these three peptides are cross-linked as well as the nature of the cross-link(s) has not as yet been determined. However, it appears that neither of the highly cross-linked bone collagen fractions contains β_{12}-CB1 or β_{11}-CB1 suggesting that intramolecular cross-linking does not occur in bone collagen. In addition, preliminary results indicate cross-linking in bone collagen is somewhat random in the sense that a given peptide may be cross-linked to several others. This appears to be dependent upon the positions assumed by the molecules relative to each other in the native fiber.

Cartilage Collagen - In collaboration with Dr. Sokoloff, NIAMD, studies on the collagen in human costal and articular cartilage were continued. Two parameters of collagen in human articular and costal cartilage were examined in relation to age and the presence of degenerative joint disease: the proportion of collagen in the total protein of the tissue and extractability of the collagen in a denaturing solvent, 5M guanidine hydrochloride. Collagen constitutes approximately 90% of the protein of articular cartilage at all ages studied (range: early infancy, 3 mo., to 90 yrs.). Only 1% of the collagen of normal articular cartilage could be solubilized by prolonged extraction with 5M guanidine. This was true of even the youngest specimens examined suggesting that cross-linking between the collagen molecules in this tissue is widespread and occurs rather rapidly. The solubility of the collagen in severely fibrillated articular cartilage samples was slightly elevated, approximately 1.5% of the total collagen being solubilized in 5M guanidine. This increased solubility of the collagen in fibrillated specimens is not considered significant with regard to the severity and extent of the disease process.

In costal cartilage, by contrast, there were age-correlated changes in the content and extractability of the collagen. The proportion of noncollagenous protein in the costal cartilage increased almost threefold during the years of skeletal growth and then reached a steady value. Although the solubility of collagen in costal cartilage remained quite low, it was observed that approximately 5.0% of the total collagen could be extracted by 5M guanidine from young specimens. Less than 1% of the total collagen could be extracted from costal cartilage samples removed from individuals over 30 years of age.

Since the studies on cartilage collagen in human samples were somewhat limited because of the extremely low solubility characteristics of the collagen in these tissues, an investigation of the collagen in chick cartilage was initiated. Our effort has been concentrated on sternal cartilage of the young chick since this tissue offers a relatively large mass of cartilage uncontaminated with bone. Moreover, histological examination and election microscope observations indicate that the collagen fibers of this tissue possess the characteristics usually seen for cartilage collagen, viz., slender fibers in which the usual 680A periodicity is not observed. Initial studies indicate, somewhat surprisingly, that the best solvents for extraction of collagen from cartilage samples are neutral salt solutions. Although 0.5 M acetic acid is effective in removing up to 20% of the collagen from the bones of lathyritic chicks, this solvent is completely ineffective in regard to sternal cartilage from the same animals. Extraction of the cartilage samples with 1.0M sodium chloride at neutral pH is approximately as effective in solubilizing collagen from the cartilage samples as acetic acid is with bone samples. These results have been interpreted as indicating that at low pH cationic collagen molecules are prevented from passing into solution in the presence of the large proportion of negatively charged chondroitin sulfate molecules found in cartilage, whereas reduction of the overall positive charge of collagen at neutral pH reduces the attraction for sulphated polysaccharides. Characterization of the CNBr peptides of both soluble and insoluble cartilage has begun. Preliminary results indicate that the peptides derived from cartilage collagen are different from those found in bone collagen suggesting that cartilage collagen possesses a primary structure different from that of bone collagen. This finding may in part explain the different morphology of collagen fibers in cartilage and indicates the presence of multiple genes for collagen synthesis.

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. In addition, it is expected that the techniques being developed to examine insoluble collagens at the level of CNBr peptides will have wide applicability to the study of collagen in clinical samples in which a variety of connective tissue diseases are manifest.

Part B

Publications:

Miller, E. J., van der Korst, J. D., and Sokoloff, L: Collagen of Human articular and costal cartilage. Arthritis and Rheumatism, 12: 21-29, February 1969.

Eanes, E. D., and Miller, E. J.: Effect of Covalent Cross-linking on the x-ray Diffraction Properties of Chick Bone and Rat Tail Tendon Collagens. Arch. Biochem. Biophys. 129: 769-771, February 1969.

Miller, E. J., Lane, J. M., and Piez, K. A.: Isolation and Characterization of the Peptides Derived from the 1 Chain of Chick Bone Collagen after Cyanogen Bromide Cleavage. Biochemistry, 8: 30-39, January 1969.

Lane, J. M. and Miller, E. J.: Isolation and Characterization of the Peptides Derived from the 2 Chain of Chick Bone Collagen after Cyanogen Bromide Cleavage. Biochemistry, 8,2134, 1969.

Serial No. NIDR-56 (68)
1. Biochemistry
2. Protein Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Hormone Receptors

Previous Serial Number: None

Principal Investigator: Dr. M. M. Rubin

Other Investigators: None

Cooperating Units: Dr. B. W. O'Malley, Endocrinology Branch, NCI

Man Years:

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

Project Description:

Objectives:

It is believed that hormones act by binding to specific receptor macromolecules which are then able to influence some cellular event. It is the purpose of this study to characterize the progesterone receptor in chick oviduct.

Methods Employed:

A single administration of progesterone to estrogen-stimulated chickens induces the synthesis of a specific protein, avidin. The induction can be done in the whole animal, in tissue culture, or in minced oviduct. Fractions from these materials are taken to study binding of labeled progesterone utilizing standard biochemical and physical chemical techniques.

Major Findings:

It has been shown that the cytoplasmic supernatant of homogenates of oviduct contain at least one macromolecule that binds progesterone. It sediments in sucrose gradients at about the same rate as a receptor protein found in plasma but can be distinguished from it by molecular sieve chromatography. Preliminary studies have been made of the stability of the receptor as a function of temperature,

ionic strength, pH and the presence of metals and reducing agents. The specificity of binding has been shown by competitive studies with related steroids.

Significance to Dental Research:

Hormones serve as part of a major control mechanism that maintains homeostasis and directs the production of specific products required by the body at specific times. This control mechanism is critical to the proper functioning of all the tissues of the body. How hormones operate at the molecular level is a fundamental problem that is not understood. A solution to this problem will lead to a better understanding of normal and pathological states and hopefully to a rational treatment of certain diseases.

Proposed Course of Project:

The studies will continue along the lines already established. It is hoped to more completely establish the specifity of the receptor, to partially purify it, to look for conformational changes that may play a role in the function of the hormone-receptor complex, and to study the effect of the complex on specific processes such as the steps in protein synthesis.

Part B: Not included

Serial No. NIDR-57 (68)
1. Biochemistry
2. Protein Chemistry
3. Bethesda, Md.

PHA-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Characterization of Muscle Proteins

Previous Serial Number: None

Principal Investigator: Dr. E. F. Rossomando

Other Investigators: Dr. K. A. Piez

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To characterize the proteins of muscle and study their mode of action. The immediate problem is to purify myosin and determine its molecular weight and chain structure.

Methods Employed:

Usual laboratory procedures

Major Findings:

One of the major problems in studying myosin is that it polymerizes irreversibly on standing in solution. It has been found possible to chromatograph myosin on molecular sieve columns and prepare pure monomer free of both high and low molecular weight contaminants. Myosin prepared this way was found to be homogeneous in the ultracentrifuge and to have a molecular weight of 450,000.

Significance to Dental Research:

The proteins of muscle interact with each other and with low molecular weight compounds to convert chemical to mechanical energy.

An understanding of the process is fundamental to an understanding of body movement and of diseases that affect muscles. An important first step is to characterize the proteins of muscle.

Proposed Course of Project:

The characterization of myosin will continue. The next step is to denature the protein and determine the number and type of polypeptide chains present.

Part B: Not included.

Serial No. NIDR-58 (62)
1. Biochemistry
2. Connective Tissue
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: The Chemistry and Biosynthesis of Connective Tissue

Previous Serial Number: NIDR-22

Principal Investigator: Dr. G. R. Martin

Other Investigators: Dr. E. Schiffmann, Dr. E. R. Goldstein and Dr. R. C. Siegel

Cooperating Units: Kaiser Medical Research Institute, San Francisco, California

Man Years:

Total:	7
Professional:	3
Other:	4

Project Description:

Objectives:

The purpose of this project is to determine the steps involved in the formation of normal connective tissues. Particular emphasis is placed on the chemistry of collagen and elastin and the enzymatic step in the cross-linking of these proteins. In addition, the synthesis of collagen in normal and diseased states is being studied.

Methods Employed:

Usual laboratory techniques with the exception that fibroblasts are grown from skin biopsies obtained from normal subject and patients with various inheritable disorders of connective tissue.

Major Findings:

We have recently described the presence of an enzyme lysyl oxidase, in extracts of connective tissue that converts lysine in peptide linkage to α-aminoadipic-δ-semialdehyde. This is presumed to be the specific enzymatic step in collagen as well as elastin cross-

linking. Other work has established that collagen can cross-link spontaneously after the lysine-derived aldehyde is formed. Following the incubation of highly radioactive collagens with purified preparations of the enzyme, we have been able to reisolate the collagen and demonstrate the formation of α-aminoadipic-δ-semialdehyde in the α chains that compose the molecule. In addition, we have observed the enzyme-dependent cross-linking of an αI with an α2 chain to form the β_{12} component. α-aminoadipic-δ-semialdehyde formation and the cross-linking of α chains is blocked by the lathyrogen β-aminopropionitrile which inactivates lysyl oxidase. The helical structure of the collagen molecule is not necessary for recognition since denatured collagen is also a substrate. Studies with various lysine peptides which inhibit lysyl oxidase suggest that the presence of an acidic and an aromatic amino acid in peptide linkage near the lysine to be oxidised are important factors determining enzymatic recognition. When examined on molecular sieves the enzyme activity elutes as a single peak with a molecular weight somewhat higher than 70,000. Studies with various chelators and copper deficient animals indicate that copper is a cofactor for the enzyme.

In collaboration with scientists at the Kaiser Research Institute, we have determined the distribution of antigenic sites in the chains of rat and guinea pig skin collagen. Antibody formed following the injection of rat or guinea pig skin collagen are species specific. Antibody is directed primarily against the α2 chain of the collagen with little directed against the α1 chain. β_{12} contains an additional antigenic determinant which is presumably in the cross-linked portion at the N-terminal end of this component. These findings are in accord with recent studies on the amino acid sequences in the N-terminal region of the α1 and α2 chains from various species, which show great similarities in the α1 chains and marked differences in α2.

Fibroblasts grown from skin biopsies have been found to synthesize and cross-link collagen. Cross-linking can be blocked by the addition of β-aminopropionitrile to the media in which the cells are grown, allowing the extraction and further characterization of the collagen. About 80% of the collagen produced by fibroblasts from normal subjects is found precipitated. The media contains an excess of the α2 chains suggesting that it is either produced in excess or less efficiently utilized than α1 in the synthesis of the collagen molecule. The precipitated collagen synthesized by some fibroblasts cultures contains far more α1 than would be expected from a collagen with the composition of 2 α1 and 1 α2. The media from these cultures contains a hydroxy-proline-containing protein with the solubility properties of collagen, that contains an excess of α2 chains. These findings suggest that collagen molecules are synthesized from a pool of chains and that molecules

with differing chain composition are formed. Much more of the collagen synthesized by fibroblasts from patients with Marfan's and the Ehlers-Danlos syndrome is found in the media than normal indicating that in these conditions collagen is not precipitated as efficiently as normal fibroblast cultures. This may explain the fragility of connective tissues in these conditions.

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:

Further studies on the enzymatic step in collagen cross-linking are planned in order to define the enzyme, lysyl oxidase, and the mechanism by which lathyrogens inactivate it. Possible relation of lysyl oxidase activity to various disease conditions is planned. Further characterization of collagen synthesis by fibroblasts from normal subjects and patients with various diseases is planned.

Part B

Publications:

Schiffmann, E., Martin, G. R., and Miller, E. J.: Matrices that calcify. Chapter in a book on Biological Calcification, edited by Prof. Harald Schraer, Department of Biophysics, University of Pittsburgh, Appleton, Century Crofts, in press.

Pinnell, S. R., Martin, G. R., Miller, E. J.: The nature of the inhibition of desmosine biosynthesis by D-penicillamine. Science 161;475-476, 1968.

Miller, E. J. and Martin, G. R.: The chemistry of bone collagen. Clinical Orthopaedics and Related Research 59:195 (1968).

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. Nat. Acad. Science 61:708-714 (1968).

Martin, G. R. and Pinnell, S. R.: Cross-linking of collagen and elastin: an enzymatic step. In A. Bertelli and J. C. Houck. Proceedings Conf. on Inflammation Biochemistry, Excerpta Medica. In press. 1969.

Serial No. NIDR-59 (62)
1. Biochemistry
2. Connective Tissue
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Calcification of Organic Matrices

Previous Serial Number: NIDR-23

Principal Investigator: Dr. E. Schiffmann

Other Investigators: None.

Cooperating Units: None.

Man Years:

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

Project Description:

Objectives:

To study basic mechanisms whereby organic matrices calcify. The system under investigation is elastin-containing tissue in vitro.

Methods Employed:

Elastin Tissue: Rat aortas from male Sprague-Dawley animals were incubated in serum for 24 hours in the presence of varying amounts of fluoride. Mineralization of the matrix was measured by uptake of Ca^{45} which was added to the serum prior to incubation. Incubated aortas were washed, digested 24 hrs in 0.1N HCl at 25°, and aliquots of the digest counted in a scintillation spectrometer.

Major Findings:

It has been reported that the aortas of persons from areas where the waters contain fluoride are less heavily calcified than those where fluoride is low. As a model system, we have studied the effect of fluoride on the mineralization of rat aortas incubated in a metastable solution of calcium and phosphate. In the presence of the aorta, calcium and phosphate precipitate and are deposited

in the aorta, whereas in the absence of the aorta there is no precipitation. Addition of fluoride (1-8 ppm) progressively inhibited (up to 80%) mineral deposition. This effect of fluoride could not be attributed to the formation of a calcium fluoride complex. Rather, analysis of the initial precipitate indicated that the first phase forming on the aorta corresponded to material similar to octacalcium phosphate which later converted to hydroxyapatite. Fluoride is known to inhibit the formation of octacalcium phosphate. These findings suggest that fluoride prevents the mineralization of non-osseous tissue by preventing the formation of the initial, more readily precipitating mineral phase.

Significance to Dental Research:

Processes whereby osseous and non-osseous tissues mineralize are not well understood. Studies on *in vitro* mineralizing systems such as elastin-containing matrices may contribute to understanding both normal and pathological calcification.

Proposed Course of Project:

Elastin: It is felt that the *in vitro* studies on elastin-containing matrices have been taken as far as one can in producing fruitful results on calcification. This approach is therefore concluded.

Part B

Publications:

Schiffmann, E., D. R. Lavender, E. J. Miller, & B. A. Corcoran: Amino acids at the nucleation sites in mineralizing elastin tissue. Calcified Tissues (in press) 1969.

Schiffmann, E. and B. A. Corcoran: Role of a ternary complex in nucleation. Calcified Tissues (in press) 1969.

Schiffmann, E., Martin, G. R., and Miller, E. J.: Matrices that calcify. Chapter in a book on Biological Calcification, edited by Prof. Harald Schraer, Dept. of Biophysics, University of Pittsburgh, Appleton, Century Crofts, in press.

Serial No. NIDR-60 (61)
1. Biochemistry
2. Connective Tissue
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

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

Previous Serial Number: NIDR-24

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
Mr. Harold McCann and Dr. Finn Brudevold, Forsyth Dental Center, Boston, Mass.

Man Years:

Total:	None (Research Contract)
Professional:	"
Other	"

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.

Noted are the extensions to the contract in terms of dates and funding arrangements; some of these arrangements have been terminated, but all fall within this reporting year, which includes July 1, 1968 to June 30, 1969. The extensions: PH43-66-941, Albany Controls, Grand Rapids Water Fluoridation Study, 10/20/67 - 12/31/68, $710.

PH43-66-941, Grand Rapids Water Fluoridation Study, 6/3/66 - 3/2/69, without additional funds.

PH43-66-941, Albany Controls, Grand Rapids Study, 2/28/68 - 1/31/69.

PH43-66-941, Grand Rapids Water Fluoridation Study, 5/3/67- 3/31/69.

Major Findings:

Sample collection and histological examination is well advanced and chemical analysis has begun. Most of the fluoride determinations have been performed. In addition some of the calcium and phosphate analyses have been completed. However, data are not yet sufficient for conclusions to be made.

Proposed Course of Project:

Refer to 'Methods Employed'.

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.

In addition some information might be gained on the effects of fluoridation upon vascular calcification, since mineral contents of aortas are also being determined.

Part B Not included.

Serial No. NIDR-61(69)
1. Biochemistry
2. Connective Tissue
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Studies on the Cross-linking of Collagen

Previous Serial Number: None

Principal Investigator: Dr. E. Schiffmann

Other Investigators: Dr. G. R. Martin

Cooperating Units: Dr. P. Bornstein, Univ. of Washington, Seattle, Wash.

Man Years:

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

Project Description:

Objectives:

To study the nature of the intramolecular cross-linking process in collagen and to elucidate the structure of the cross-link.

Methods Employed:

1. The cross-linking process:

Soluble collagen was prepared from skins of normal, lathyritic, and penicillamine-fed rats and then incubated in fibrous form at 37° in neutral salt solution for varying periods of time. Samples were subjected to carboxymethyl cellulose chromatography to separate α and β-components. Both whole collagen samples as well as samples of α and β-components were treated with cyanogen bromide to produce peptides. The peptides were separated and isolated with the aid of Sulfoethyl Sephadex chromatography.

2. The chemistry of the cross-link:

β-Components were isolated from acid-extracted rat skin collagen with the aid of carboxymethyl cellulose chromatography. These were treated with cyanogen bromide, and the resulting peptides separated by SE-Sephadex chromatography. The isolated cross-link-containing

peptide was reduced with sodium borohydride to stabilize it, hydrolyzed in base, and the cross-linking compound isolated with the aid of ion exchange chromatography. This material was then degraded to yield both formaldehyde and amino acids. The formaldehyde was isolated as the dimedon derivative, and the amino acids were analyzed by ion exchange chromatography.

Major Findings:

1. The cross-linking process

The first step in the cross-linking of collagen is the enzymatic deamination of certain lysyl residues to form peptide bound α-aminoadipic-δ-semialdehyde (allysine). Intramolecular cross-links are formed by the condensation of allysine residues and intermolecular cross-linking may involve similar intermediates. It was the purpose of this study to determine whether cross-linking can occur spontaneously after allysine formation. Collagen deficient in allysine was prepared from lathyritic rats and collagen with a high allysine content was prepared from rats fed penicillamine. The composition of the collagen from penicillamine fed rats changed when incubated in fibrous form in vitro. There was a decrease in the content of α chains and an increase in the content of β-components. Characterization of the cross-link containing peptide indicated that it was identical to the cross-linked peptide formed in vivo. Therefore, the formation of normal intramolecular cross-links can occur spontaneously after allysine formation and is probably not enzymatic.

2. The chemistry of the cross-link

The purpose of this study is to isolate the lysine derived cross-link and by specific chemical degradations to determine its structure. β_{12}-CB1, the cross-link containing peptide, was isolated from β_{12} after cyanogen bromide cleavage. Following reduction with $NaBT_4$ and alkaline hydrolysis a radioactive peak corresponding to the reduced aldol product was isolated. If current theories of the nature of the cross-link are correct, oxidative degradation of this compound should yield 1 mole of formaldehyde, 1 mole of α-aminoadipic acid and 1 mole of glutamic acid. Treatment of the labeled reduced aldol with osmium tetroxide and periodate or permangenate and periodate liberated approximately 60-70% of the radioactivity in the compound as formaldehyde.

Four ninhydrin reacting compounds were formed. Two were identified as aspartic acid and α-aminoadipic acid and two are not yet identified. The finding of formaldehyde and α-aminoadipic acid after degradation is consistent with the postulated structure of the aldol whereas the isolation of aspartic acid suggests that some rearrangement of the aldol had occurred. The two unknown compounds may be derivatives of glutamic and pimelic acid.

Significance to Dental Research:

Any information on the biochemical processes involved in bond formation in a connective tissue such as collagen may be related to the tensile strength of such tissues, the aging process in these tissues, the mineralization potential of collagen matrices, and certain pathological manifestations in connective tissue.

Proposed Course of Project:

It is proposed to study whether other cross-links can be formed during incubation of collagen for longer periods of time. It is conceivable that different dimeric and more highly aggregated species may be formed, which may be quite similar to the intermolecular bonds in collagen. Further characterization of the cross-link is planned.

Part B. Not included.

Serial No. NIDR-62 (52)

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

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Chemistry and Mechanism of Action of Guinea Pig Liver Transglutaminase

Previous Serial Number: NIDR-25

Principal Investigator: Dr. J. E. Folk

Other Investigators: Dr. H. Kato, Dr. S. I. Chung, and Dr. J. M. Connellan

Cooperating Units: None

Man Years:

Total:	5 1/4
Professional:	3 1/4
Other:	2

Project Description:

Objectives:

It is the long range purpose of these studies to gain a comprehensive knowledge of the basic mechanisms of enzymatic catalysis. Transglutaminase has been chosen as a model for study because it displays catalytic properties that are typical of a number of classes of enzyme i.e. a) it features an essential requirement for metal ion, b) catalyzes hydrolytic, esterolytic and transferase reactions, c) forms a stable convalent enzyme substrate intermediate complex and d) appears to be of prime physiological significance. The immediate problems under consideration are: 1) order of substrate addition and product release; 2) mode of metal ion activation; 3) enzymatic specificity; 4) functional groups involved in catalysis; and 5) overall molecular structure of the enzyme protein and its relation to catalysis.

Methods Employed:

Most recently developed methods of kinetic analysis have been applied. The enzyme has been subjected to several unique chemical modifying agents, including structural isomers of alkylating reagents. A number of isomers and analoges of glutamine substrate and of amine substrate have been synthesized and tested in an

effort to "map" the active site of the enzyme. The enzyme has been examined as a possible agent for the quantitative determination of glutamine in proteins.

Major Findings:

Kinetic and chemical findings support a "ping pong" substrate addition and product release mechanism for transglutaminase. Is this mechanism glutamine substrate adds to enzyme to form a convalent acyl enzyme intermediate with release of NH_3. Water adds with the formation of glutamic acid as the product (hydrolysis) or an acceptor amine adds with the formation of a substituted amide as product (transfer). These studies also show that the essential metal ion, Ca^{++}, functions in the acceptor amine step of transfer as well as in the initial binding of glutamine substrate.

The findings that 5,5'-dithiobis(2-nitrobenzoic acid) at the level of 1 mole per mole of enzyme, in the absence of Ca^{++}, results in a complete loss of hydrolysis and transfer activities with no loss in esterase activity and that this is accompanied by the formation of a single intramolecular disulfide bond in the enzyme protein has given some indications as to the differences in structural requirements for various enzymatic activities. The fact that this modication causes gross changes in the Ca^{++} requirements for certain changes, e.g. reactivation with reducing agents, suggests that the divalent cation binding is effected.

Reaction of transglutaminase with 2 moles per mole of sodium tetrathionate results in complete loss in transfer and hydrolysis activities and about 50% loss in esterase activity. Two intramolecular disufide bonds are formed as a result of this treatment one of which may be broken with no recovery of activities. The responsible disulfide bond appears to be different from that formed by the action of dithiobis-(nitrobenzoic acid) described above.

The irreversible inactivation of transglutaminase by D- and L-chloropropionic acid and its amide has been studied as a function of pH and Ca^{++} concentration. The pronounced differences observed with the isomers suggest an important steriochemical arrangement of functional groups at the active center of the enzyme and give evidence for involvement of a group of pK 5.5 to 6.5.

Bifunctional chemical modifying agents, e.g. dibromoacetone and α-α-dibromoadipic acid, have been prepared in an effortot identify amino acid residues in addition to an essential -SH groups of the enzyme. Preliminary studies show these agents are potent irreversible inactivators.

The use of ^{14}C-labeled D- and L- alanine ethyl esters as acceptor amines in the transfer reaction shows results that conform with above evidence of a stereochemical arrangement at the active site of the enzyme.

The finding that the methylene chain of glutamine, $(CH_2)_2$, may be lengthened, but not shortened suggests a fully extended orientation of substrate on the enzyme surface. An induced fit is indicated in the case of glutamine substrate which is the only substrate that inhibits the esterase activity of the enzyme.

Preliminary studies with the sulfydryl agent, p-mercuribenzoate, show that binding of 2 moles of reagent per mole of enzyme results in dramatic changes in enzymatic activities. The nature of these changes indicate that a single step in catalysis, probably-transfer of acyl group from enzyme to acceptor amine, has been modified. Studies are underway to define further this change and to relate the enzyme groups involved with the chemical step in catalysis.

A method has been developed for quantitatively determining the glutamine content of proteins. In this procedure oxidized protein is incubated with transglutaminase in the presence of ^{14}C - glycine ethyl ester. The incorporation of the ester, as measured by conventional counting methods, is a measure of glutamine residues.

Proposed Course of Project:

These studies will be continued. More effort will be focused toward relating the chemical and molecular structure of the enzyme protein to its catalytic activity and to an understanding of the general molecular structure of this protein.

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.

Publications:

1. Boothe, R. L. and Folk, J. E.: A Reversible, Calcium-Dependent, Copper-Catalyzed Inactivation of Guinea Pig Liver Transglutaminase, J. Biol. Chem. 244: 399, Jan. 1969.

2. Toda, H. and Folk, J. E.: Determination of Protein-Bound Glutamine, Biochim. Biophys Acta, 175: 427, March 1969.

3. Connellan, J. M. and Folk, J. E.: Mechanism of the Inactivation of Guinea Pig Liver Transglutaminase by 5,5'-Dithiobis(2-Nitrobenzoic acid) J. Biol. Chem., in press.

4. Folk, J. E.: Mechanism of Action of Guinea Pig Liver Transglutaminase VI Order of Substrate Addition. J. Biol. Chem., in press.

5. Folk, J. E.: Carboxypeptidase B (porcine pancreas) in G. Perlman and L. Lorand: Proteolytic Enzymes, in press.

6. Folk, J. E.: Chymotrypsin C (porcine pancreas) in G. Perlman and L. Lorand: Proteolytic Enzymes, in press.

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

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Exogenous and Endogenous Factors Affecting Normal Development of the Oral Facial Region

Previous Serial Number: NIDR-26

Principal Investigators: Dr. C. T. G. King and Dr. A. J. Steffek (ADA)

Other Investigators: Dr. A. C. Verrusio (ADA) and A. L. Wilk

Cooperating Units: American Dental Association
Dr. A. G. Hendrickx, Southwest Foundation for Research and Education, San Antonio, Texas

Man Years:

Total:	7
Professional:	4
Other:	3

Project Description:

Objectives:

The interest and research investigations of the Pharmacology Section, LB, NIDR, revolve about the field of mammalian organogenesis, pathogenesis and etiology of cleft palate and related problems. Having completed studies in normal organogenesis of several species of mammals our interest now has shifted to investigations of normal palatal closure in subhuman primates in order to evaluate these species as possible animal models for studies of environmentally induced abnormalities. Environmentally induced congenital anomalies, the emphasis being on cleft lip and palate, are being induced by a variety of agents in a number of mammalian species in order to study the comparative pathology of the lesion produced. The etiology of the lesions produced is being investigated from several aspects such as: metabolism and placental transfer, or blocking of the drugs used, genetic and mitochondrial studies.

Subproject A:

Mechanisms involved in normal development of the secondary palate.

Objectives:

Closure of the secondary palate involves the converging rotation of both shelves with a subsequent fusion at the line of contact. Since the two steps do not occur simultaneously, they can be considered separate phases of palatogenesis--each subject to experimental study and manipulation.

Methods Employed:

Mammalian embryos undergoing palatogenesis were obtained by laparatomy or cesarian section. The embryos were decapitated, and subsequently prepared for routine histological examination.

Major Findings:

a) Palatal Closure: Rhesus monkey (Macaca mulatta)

The histology of palate closure was characterized in the rhesus monkey. One embryo was obtained by cesarian section on each of days 40, 42, 46 and 50 of pregnancy. The palatal shelves were vertical and closely approximated the lateral borders of the tongue on day 40. Meckel's cartilage was present but no mandibular or maxillary osteogenesis had occurred. In the embryo, obtained on day 42, the palatal shelves were still directed vertically but downward growth of the shelves had given them a more elongated appearance. Mandibular ossification had also begun in the region of Mechel's cartilage. The palatal shelves in the 46-day specimen were horizontal and there was contact between them. The epithelium overlying the shelves at the nasal surface and midline was relatively continuous although minor gaps occurred at both these sites. Palate closure however was not complete since in the most posterior region of the palate, fusion had not occurred between the horizontally oriented palatal shelves. Ossification of the maxillary and vomer bones was also observed. The embryo obtained on day 50 of gestation showed a further breakdown of the nasal and midline epithelium of the palatal shelves. These results indicate that palatal development in the rhesus monkey takes place on days 42 - 50 of gestation and palate closure occurs during day 46.

b) Palatal Closure: Mechanism of Action

Last year it was suggested that rapid growth and straightening of the cranial base might play a role in palate closure by providing the "internal shelf force." This hypothesis was based on a report that the cranial base is flexed before palate closure begins, but undergoes a gradual straightening as the process of closure progresses until, finally, when the palatine processes are horizontal, it is straight (Harris, 1964).

First we confirmed the observation of Harris that there is a gradual decrease in angulation of the cranial base during morphogenesis of the palate. We then tested the proposed mechanism for palate closure in the following way. Medial sagittal sections of rat embryos treated with chlorcyclizine and 6-aminonicotinamide (potent cleft palate teratogens) were examined and the angle of flexure of the cranial base was compared with that of normal animals before, during, and after palatal closure to see if the cranial base is affected by these teratogens. The results indicate that straightening of the cranial base is delayed in those embryos treated with the teratogens. If this were to cause a delay in rotation of the shelves, as predicted by the model discussed last year, the shelves might not be able to meet in the midline and fuse when they finally become horizontal.

Significance to Dental Research:

These investigations have 1) described the palatal development in the rhesus monkey and show a close similarity in the timing of palatogenesis in this species when compared to human palatal development and 2) focused on other craniofacial structures that potentially participate in normal palate closure.

Proposed Course of Project:

a) Palatal Closure: Rhesus Monkey

The preliminary findings in the rhesus monkey will be extended to other non-human primates and an attempt will be made to produce cleft palate in the primate with specific teratogens.

b) Palatal Closure: Mechanism of Action

Mice of the A/J and C57BL/6J strains will be treated with 19 mg/kg of 6-aminonicotinamide (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--one of which is resistant is 6-AN (C57BL/6J) and the other (A/J) susceptible.

Subproject B:

Comparative oral-facial teratology in various mammals.

Objectives:

To investigate the susceptibility and comparative pathology of cleft palate induced by specific teratogenic agents in various mammalian species.

Methods Employed:

Fetuses obtained from lathyrogen-treated pregnant mice, rats, rabbits, ferrets and baboons, and from 6-aminonicotinamide treated rodents, and thalidomide treated ferrets during palate development were observed for gross malformations. The heads were subsequently removed, fixed, embedded and sectioned by routine techniques for the histological characterization of the oral-facial lesions.

Major Findings:

a) Lathyrogenic agents:

The teratogenesis of experimentally-induced cleft palate has been studied in this laboratory with a number of environmental agents in a variety of mammalian species. Recently our direction has been focused on cleft palate production with lathyrogenic agents. A high incidence of cleft palate has been shown in A/J mice and Sprague-Dawley rat fetuses after maternal ingestion of a 50:50 Lathyrus odoratus sweet pea diet. Other lathyrogens (BAPN, AAN, D-penicillamine and semicarbazide) administered during various stages of organogenesis were also effective in producing a high incidence of cleft palate in rats. Cyanoacetic acid, a major metabolite of BAPN in doses from 1000- to 100 mg and given to pregnant rats during varied intervals of pregnancy, did not produce cleft palate in over 150 viable offspring examined. The susceptibility of rabbit embryo to the teratogenic action of BAPN and semicarbazide was also investigated. Pregnant rabbits, treated with 3-5 gms of BAPN during days 14-19 (palate closure in the rabbit is day 17) produced a high incidence of fetal resorption but less than 20 percent cleft palate. A more effective teratogen was semicarbazide in this species which at 200 mg, given days 15-19, produced over 90 percent cleft palate rabbit fetuses.

The susceptibility of ferrets to the teratogenic action of BAPN was also investigated. BAPN, 400 mg given days 20-28 of gestation (palatal closure occurs during day 27) produced a high incidence of fetal resorption and cleft palate (80%) in the viable offspring.

In a collaborative program with Dr. A. G. Hendrickx, Chairman, Dept. of Anatomy, Southwest Foundation for Research and Education, San Antonio, Texas, the teratogenic potential of BAPN and AAN was assessed in nine pregnant baboons (Papio sp.)

Timed pregnancies were based on single matings of approximately 8 hours and the day of mating considered as day 0 of pregnancy. The drugs were administered orally, concealed in an apple or banana, intravenously or intramuscularly during varied intervals between days 37-50 of gestation. The fetuses were obtained by cesarean section and examined for gross congenital malformations. The results of these initial studies are presented in the following table.

Dose mg/kg/day	Days of Treatment (Gest. Age)	Results
BAPN		
300 (ORAL)	37 - 48	Fetal resorption
500 (IV)	43 - 48	Fetal maceration
AAN		
20 (IM)	38 - 50	Abortion
60 (ORAL)	38 - 48	Fetal resorption
75 (ORAL)	43 - 48	Malformations of the shoulder and digits of the right foot
75 (ORAL)	43 - 48	Twins - Both with abnormal curvature of arms and legs
130 (ORAL)	46 - 48	Normal
130 (ORAL)	45 - 48	Normal
60 (ORAL)	40 - 48	Normal

These findings show that specific lathyrogens effectively produce cleft palate in mouse, rat, rabbit, ferret and perhaps the baboon. No agent has produced experimental cleft palate in as many varied species as have the lathyrogens that have been tested in this laboratory.

b) Thalidomide:

Pregnant ferrets received 100 mg thalidomide orally, days 10-20 of pregnancy. The viable fetuses that were obtained after this treatment had a low incidence (10%) of limb anomalies and cleft palate. As the dosage of thalidomide was increased to 200 mg and given orally, days 10-20, the limb anomalies and cleft palate again were observed. A moderate incidence (20%) of uni- or bilateral cleft lip was also seen in viable ferret fetuses. This latter lesion has been exceedingly difficult to produce experimentally to date in any species. In fact, no report to our knowledge has even demonstrated experimental cleft palate/lip malformations in any species after thalidomide treatment. These malformations have not been observed in over 150 viable control ferret fetuses. These results indicate that the ferret is also susceptible to thalidomide --an agent to which most experimental animals are resistant.

Significance to Dental Research:

a) Lathyrogens

The predicted ability to produce specific types of palatal lesions in various mammals offers an excellent technique in studying underlying mechanisms involved with experimental production of cleft palate. Also the potential production of cleft palates in subhuman primates would be of interest both from the viewpoint of

etiology and as experimental models for the evaluation of operational procedures applicable to human surgery.

b) Thalidomide

The ability to produce cleft lip with thalidomide offers a promising experimental model in which to study this oral-facial malformation, which in the past has been most difficult to produce.

Proposed Course of Project:

a) Lathyrogens - The histological characterization of the oral-facial lesions produced in rabbits and ferrets will be compared to the oral pathogenesis that has been obtained in rats. In addition based on the teratological data obtained with baboons in the preliminary s udy, lathyrogens will be tested for potential cleft palate production (AAN and BAPN). The pregnant baboons will be treated from days 38-50, which encompasses the time palate closure. BAPN will be administered at a dose level of 200 mg/kg because 300 mg/kg previously produced fetal resorption. AAN will be administered at dose levels of 50-75 mg/kg doses to duplicate and potentially extend the teratology of the initial studies.

Experiments will be designed in an attempt to determine what factors are responsible for the difference in timing of administration of the compounds. In the case of benzhydrylpiperazines the 14th day is critical while under the present situation 15 is.

b) Thalidomide

The preliminary investigations will be extended to increase the frequency of experimental cleft lip/palate production in the ferret with thalidomide.

Subproject C:

Studies on the etiology of experimentally induced congenital malformation.

Objectives:

To elucidate the mechanisms of action in the induction of malformations of the oral facial region.

Methods Employed:

a) It has clearly been demonstrated that BAPN is a teratogen that is responsible for the production of cleft palate in both the young of pregnant rats and rabbits; however, the amount of BAPN required for the rabbit is much greater than for the rat. The principal metabolite of BAPN is cyanoacetic acid, which, by itself

is not teratogenic in the rat, for this reason, it was decided to determine the levels of BAPN and cyanoacetic acid in maternal and fetal tissue after administration of BAPN. Simple and sensitive methods for the determination of BAPN and cyanoacetic acid in animal tissues were developed in this laboratory that yielded quantitative results when the threshold of BAPN was 20 gamma/gm of tissue and cyanoacetic acid. 10 gamma/gm of tissue in the original sample.

b) Mitochondria were obtained from 6-aminonicotinamide treated A/J and C57BL/6J mice and compared by sucrose density centrifugation.

c) To determine if the teratogenic activity of norchlorcyclizine analogs can be predicted by their relative ability to bind in vitro and displace Ca from a suspension of bovine nasal septum. Structural analogs of norchlorcyclizine were tested for teratogenic activity by intrauterine application over 13-day rat embryos. This procedure allows direct presentation of compounds to the fetus and so eliminates the interferences of maternal metabolism and placental transfer. In addition it narrows down the critical time in organogenesis during which the drug or analog would be effective and finally it is also of great value in determining the minimal functional structure of the compound under investigation.

These same analogs were also tested for in vitro cartilage binding affinity by addition to suspensions of bovine nasal septum at pH 7.0.

Major Findings:

a)
Oral administration of BAPN (500 mg/rat) on day 15 in pregnant rats resulted in 100% congenital malformations. Six hours after administration on day 15, the maternal liver contained 200 ± 18 μg/g BAPN and 507 ± 145 μg/g cyanoacetic acid.

Preliminary experiments demonstrate that the rabbit is extremely resistant to the teratogenic effects of BAPN. A dose of 3 gms/animal has to be reached before any results are obtained. Metabolic studies carried out 6 hours after administration of this dose of the drug on day 16 of pregnancy demonstrated that the maternal liver contained 120 μg/g cyanoacetic acid and the fetus contained 284 ± 28 μg/g cyanoacetic acid. In contrast these tissues had only trace amounts of BAPN.

These results indicate that the rabbit is more resistant to the teratogenic effects of BAPN and that it metabolized it at a much faster rate than the rat, leading to the accumulation of its non-teratogenic metabolite cyanoacetic acid.

b) Maternal treatment with 6-AN produces a higher frequency of

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cleft palate in the offspring of A/J than C57BL/6J mice. The F_1 hybrids of crosses between these strains show a matroclinous reciprocal-cross difference in the frequency of induced cleft palate which persists when the two types of F_1 female (AC from A/J ♀ x C57♂ and CA from C57 ♀ x A/J ♂) are backcrossed to the A/J strain. This suggests that the strain difference in 6-AN-induced cleft palate is determined in part by factors transmitted through the egg cytoplasm and 6-AN forms an inactive NAD analogue that interferes with oxidative phosphorylation in mitochondria, it was postulated that the strain difference in susceptibility to 6-AN resides in the mitochondria.

Recent studies have shown that variation in the chemical composition of mitochondria affects their density as determined by centrifugation in sucrose gradients, so, mitochondria from A/J and C57BL/6J mice were compared on density gradients. Mitochondria were extracted from adult liver and placed on linear sucrose gradients (1.35M - 1.9M). The gradients were centrifuged in the SW 41 rotor of the Backman Model L-2 for 150 minutes at 30,000 rpm. The density of A/J mitochondria was lower than that of C57BL/6J mitochondria in all cases. When mice were pretreated with 6-AN for varying periods of time before the liver homogenates were prepared, a gradual decrease in mitochondrial density was observed in both strains. After 15 minutes pretreatment with 6-AN, C57BL/6J mitochondria were noticeably lighter than those from untreated mice. However, A/J mice required a one-hour pretreatment with 6-AN before their mitochondria showed a comparable shift in density. Thus, there appears to be a strain difference in the length of time required by 6-AN to act on the liver mitochondria of these mice. Preliminary results of an electron microscopic study indicate that the mitochondria of the two strains show a similar morphological reaction to 6-AN treatment. The mitochondria of both strains swell, but C57-BL/6J mice seem to be affected sooner.

c) The compounds that induced cleft palate and malformations of the left forelimb were: norchlorcyclizine and norhomochlorcyclizine (30%); normethylcyclizine, homochlorcyclizine, and norcyclizine (10%); chlorcyclizine, methylcyclizine, chlorcyclizine-N-t-butyl, and cyclizine (<5%). Chlorcyclizine-N-oxide, chlorbenzylpiperazine, chlorbenzhydrol, and piperazine did not produce malformation. Although most of the nitrogen compounds were capable of electrostatically binding to cartilage, their binding strengths varied according to cation concentration. Thus when Ca^{2+} was excluded from the reaction tube, the amines were bound at about 10. mEq N^+/g of cartilage; however, when 0.25 mg Ca^{2+} was added with 5 mg of norchlorcyclizine, chlorcyclizine, or chlorbenzylpiperazine, the amines were bound at .85, .60, and 0 mEq N^+/g. When 1.0 mg Ca^{+2} was added, these amines were bound at .31, .11, and 0 mEq N^+/g. These results demonstrated a good parallelism between the compound's

teratogenic activity and its relative ability to bind with cartilage.

Significance to Dental Research:

a) BAPN. Studies of the difference in metabolism of a specific chemical compound such as BAPN, that induces oral facial anomalies in a variety of species, should lead to a better understanding of the etiology of the malformation.

b) The experiments on liver mitochondria demonstrate that the strain difference in response to the teratogen 6-aminonicotinamide resides in the mitochondria of the mice. Furthermore, this mitochondrial difference when defined will be the first mitochondrial mutant identified in mammals.

c) Direct access to specific anatomical areas of the developing fetus with minimal amounts of suspected teratogenic moiety can greatly aid in understanding of the pathogenesis of the malformation. The data obtained from the in vitro binding to cartilage and calcium displacement by norchlorcyclizine demonstrates a correlation between this binding affinity and the expression of teratogenicity by specific drugs.

Proposed Course of Project:

a) Metabolism studies of BAPN and possibly other lathyrogens will be continued in order to determine critical levels of the compounds or their metabolites that are required to cross the placental barrier in order to induce congenital malformations. In addition "binding" studies will be carried out in order to determine why this compound is so extremely specific, as far as timing of administration is concerned.

b) 6-Aminonicotinamide:

The electron microscopic study will be continued and expanded to include hybrid animals. An attempt will be made to find the minimum time required by both strains to show a reaction to 6-AN treatment.

The DNA of liver mitochondria from A/J and C57BL/6J mice will be extracted and placed on cesium chloride gradients. The genetic crosses indicate the presence of a possible mitochondrial mutant, and this would show if the mitochondrial DNA of the two strains is different.

c) The binding sludies of norchlorcyclizine analogs will be extended to the in vivo embryonic situation. Experiments have shown that oral administration of chlorcyclizine (120 mg/kg) to pregnant rats on day 12 induces 50% cleft palate while the same treatment on day 13

produces only 3%. However when these treatments were supplemented with a nonteratogenic dose of calciferol (Vit. D_2) on day 14, no cleft palate was produced after 12 day chlorcyclizine treatment but 60% cleft palate was realized after day 13 treatment with chlorcyclizine. Total fetal concentrations of norchlorcyclizine (the major teratogenic metabolite) were not affected by the calciferol treatment. These regimes give a good opportunity for determining if the different teratogenic responses parallel the in vivo embryonic binding of norchlorcyclizine. A dialysis technique is being developed for determining free and bound norchlorcyclizine. Other embryonic perimeters to be examined are free and bound Ca and Vit. D_2 levels.

Part B

Publications:

Steffek, A. J., Fabiyi, A. and King, C. T. G.: Chlorcyclizine Produced Cleft Palate in the Ferret (Mustela putorius furo).Arch. oral Biol. 13:1281-1283, 1968.

Steffek, A. J., King, C. T. G. and Wilk, A. L.: Abortive Effects and Comparative Metabolism of Chlorcyclizine in Various Mammalian Species, Teratology 1:399-406, 1968.

Steffek, A. J., Verrusio, A. C. and King, C. T. G.: The Histology of Palatal Closure in the Rhesus Monkey (Macaca Mulatta). Teratology 1:425-429, 1968.

Koziol, C. A. and Steffek, A. J.: Acid Phosphotase Activity in Palates of Developing Normal and Chlorcyclizine Treated Rodents. Arch. oral Biol. 14:317-321, 1969.

Wilk, A. L.: Production of Fetal Rat Malformations by Norchlorcyclizine and Chlorcyclizine after Intrauterine Application. Teratology 2:55-66, 1969.

Barrow, M. V., Steffek, A. J. and King, C. T. G.: Thalidomide Syndrome in Rhesus Monkey. Folia Primatologia 10:195-203, 1969.

Serial No. NIDR-64 (62)
1. Biochemistry
2. Cell Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Cell Growth Studies in Normal and Abnormal Subjects

Previous Serial Number: NIDR-28

Principal Investigator: Dr. H. L. Cooper

Other Investigators: Dr. S. Handmaker, Dr. J. E. Kay (Guest Worker), Dr. J. W. Graef

Cooperating Units: None.

Man Years:

Total:	6 1/2
Professional:	3 1/2
Other:	3

Project Description:

Objectives:

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 the relationship between cell growth and ribosomal RNA production.

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.

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. No drugs or other procedures beyond drawing of blood were used for our studies. No patient risk was incurred.

Major Findings:

1. A. A technique has been perfected and critically tested for differentially extracting various classes of RNA from intact lymphocytes. This permits study of movement of RNA from nucleus to cytoplasm without recourse to mechanical methods of cell disruption.

   B. I. Using the technique noted above, a detailed study of the production of the two ribosomal RNA types (28s and 18s RNA) was undertaken. It was found that in resting (non-growing) lymphocytes about half of the newly made 18s RNA is rapidly degraded to the same degree, but more slowly. Shortly after stimulation with phytohemagglutinin (PHA), which induces growth in lymphocytes, this wastage of ribosomal RNA is abolished. It is proposed that the wastage of ribosomal RNA in non-growing lymphocytes is part of a control mechanism which prevents the cells from growing. Upon stimulation with PHA, the wastage is reversed and accumulation of ribosomes ensues. This is essential for increased protein synthesis and normal cell growth.

   II. In a study of the effect of cycloheximide, which inhibits protein synthesis, on lymphocytes, it was found that in either resting or rapidly growing lymphocytes, exposure to cycloheximide almost entirely abolishes the synthesis of 18s ribosomal RNA, but not of 28s RNA. Since both 18s and 28s RNA are produced from the same precursor molecule (45s RNA), this is interpreted to mean that the 18s molecules are synthesized and rapidly degraded again. It is proposed that one or more proteins are required to be continuously produced which

normally act to protect newly made 18s RNA from degradation. Cycloheximide treatment, by preventing production of this protein, abolishes the protection of newly synthesized 18s RNA and it is rapidly degraded. It is further suggested that this protein(s) is involved in the control mechanism proposed in section 1. B. I, above and regulates the survival and accumulation of 18s RNA by being always produced in limiting quantities.

C. Several other studies of early metabolic changes during the shift of resting lymphocytes to a condition of rapid growth have been carried out.

I. Very rapidly after stimulation with PHA there is a marked increase in the production of a type of cytoplasmic RNA which may be a precursor to transfer RNA.

II. PHA treatment produces a rapid increase in the rate of phospholipid turnover in lymphocytes. This is independent of changes in either RNA or protein synthesis, and suggests that a primary phenomenon in PHA stimulation may be an effect on the cell membrane.

III. The binding of PHA to lymphocytes has been studied and the following observations made:

a. Binding is complete in 2 hours.
b. Binding will occur even with dead cells.
c. Binding is more rapid at 37° than at 4°C, although cell metabolism is not involved.
d. Binding is inhibited by serum.
e. After completion of binding to live cells, PHA effect can still be reversed for several hours by the use of anti-PHA antiserum. Subsequently the effect becomes irreversible.

These findings indicate that PHA binding and irreversible cell stimulation are separate steps.

IV. Very low doses of actinomycin-D were found to preferentially inhibit the production of 28s RNA, while 18s RNA synthesis was less strongly inhibited. This observation, together with the previously noted effect of cycloheximide on 18s RNA synthesis, indicate that potential control at the maturational level exists for both forms of ribosomal RNA.

D. The activity of RNA polymerase in resting lymphocytes and in lymphocytes following PHA stimulation is being studied. Findings to date suggest that RNA polymerase activity does not increase for some time after PHA stimulation, but that existing, inactive, molecules are activated, possibly through increased availability of DNA templates.

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.

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 alerations 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.

Part B

Publications:

1. Cooper, H., and Kay, J.: Differential extraction of RNA from intact lymphocytes. Biochim. Biophys. Acta 174: 503.

2. Cooper, H.: Studies on early biochemical changes in phytohemagglutinin-treated lymphocytes. In: Rieke, W. O. (Ed.). Proc. of 3rd Annual Leukocyte Culture Conference. Appleton-Century-Crofts. 1969 pp. 623-637.

3. Cooper, H.: Alterations in RNA metabolism during the shift from resting state to active growth. In: Baserga, R., (Ed.). The Biochemistry of Cell Division. C. C. Thomas, 1969, p. 91.

4. Cooper, H.: Ribosomal RNA production and growth regulation in human lymphocytes. J. Biol. Chem. 244: 1946, 1969.

5. Kay, J. E.: Phytohaemagglutinin: An early effect on lymphocyte lipid metabolism. Nature 219: 172-173, 1968.

6. Kay, J. E., Leventhal, B. G., and Cooper, H. L.: Effects of inhibition of ribosomal RNA synthesis on the stimulation of lymphocytes by phytohaemagglutinin. Experimental Cell Research 54: 94-100, 1969.

Serial No. NIDR-65 (69)
1. Biochemistry
2. Cell Biology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: "Studies in Cellular Immunology"

Previous Serial Number: None

Principal Investigator: Dr. J. J. Oppenheim

Other Investigators: Dr. R. Seeger, and Dr. T. C. Francis

Cooperating Units: Dr. J. B. Block, (NCI), Dr. B. Zbar (NCI), Dr. R. M. Blaese (NCI), Dr. T. A. Waldmann (NCI), Dr. W. Terry (NCI), Dr. M. Frank (NCI), Dr. R. Asofsky (NIAID), Dr. G. Goldstein (NIAID), Dr. D. E. McFarlin (NINDS), Dr. D. E. Thor (DBS).

Man Years:

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

Project Description:

Objectives:

Investigate the cellular interations between immunologically active cells such as macrophages, plasma cells and lymphocytes. Study the in vitro as well as in vivo effects of their products including antibodies, migration inhibition factor, lymphotoxic products, transfer factor and ribonucleic acid containing active extracts. Relate these in vitro cellular reactions to antigens and other triggers of lymphocyte proliferation to in vivo immunological phenomena. A study of the relationship of lymphocyte growth response in vitro to aspects of immunological phenomena in vivo by comparing the response of patients with various immunological defects to normal controls.

Methods Employed:

Lymphocytes for studies of *in vitro* proliferation 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, or by density gradients centrifugation.

The cells were stimulated to grow by addition either of phytohemagglutinin (an extract of the kidney bean) or of specific antigens. Morphological and metabolic changes were studied during the course of the subsequent cell enlargement and division.

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, anit-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 and macrophages obtained from peritoneal exudates of guinea pigs 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:

The mechanism of action and immunological relevance of *in vitro* lymphocyte proliferation induced by antigens, antibodies, antigen-antibody complexes, and nonspecific stimulants is under investigation. To broaden our understanding of these phenomena the effects of these stimulants on the lymphocytes of guinea pigs and of patients with a variety of immunological problems is being studied. We have found that patients with Wiskott-Aldrich syndrome have impaired *in vitro* lymphocyte transformation with antigens. Patients with ataxia-telangiectasia also manifest this defect, but their impaired *in vitro* lymphocyte proliferation can be partially corrected by cultivating their lymphocytes in plasma from normal

human donors. Lymphocytes from subjects with rheumatic heart disease, and aphthous stomatitis are specifically hyporesponsive to antigens from Group A hemolytic and 2A nonhemolytic streptococci respectively. These organisms have been implicated in the etiology of both the latter diseases.

The sera from some patients with cryoglobulinemia, and rheumatoid arthritis supresses in vitro lymphocyte proliferation more than control sera.

Guinea pigs with experimental autoimmune thymitis whose thymus glands are infiltrated by mononuclear cells, also show an increased proportion of lymphocytes that can be stimulated with PHA in their thymus gland.

In an inbred animal model, we are studying the mechanism of antigen processing by macrophages. Macrophages added to lymphocyte suspensions from antigen-sensitized guinea pigs caused a marked increase in proliferation of those lymphocytes when exposed to the antigen in vitro. Macrophages from either immune or non-immune animals are equally effective, but the effect was obtained only with lymphocytes from immunized animals. It was concluded that macrophages facilitate the immune response of previously committed lymphocytes to added antigen.

We are also studying the effects of polycations and polyanions on the induction of lymphocyte proliferation. Polycations were found to inhibit lymphocyte proliferation. Polyanions reversed this effect. The effect on in vitro lymphocyte responses of immunologically active materials such as poly IC and thymosin have been found not to be significant.

It has been found that complexing antigens to antibody enhances their stimulating effect in "unsensitized" adults, and immunized guinea pigs. The specificity as well as mechanism of this phenomenon is being studied. Immunologically naive lymphocytes from human newborn do not transform with these complexes indicating that prior sensitization is requisite for their stimulating effects. In contrast antibody in excess inhibits in vitro lymphocyte proliferation suggesting that antibodies modulate lymphoproliferative reactions.

Significance to Dental Research:

The role of various types of immunocompetent cells in the mechanisms of normal inflammatory responses involved in host defense and in disease states involving oropharyngeal tissues such as aphthous stomatitis, and gingival inflammation are intimately concerned with dental research.

Proposed Course of Projects

We plan to continue to study the role of macrophages and lymphocytes in immunological reactions such as delayed hypersensitivity, as a means of understanding autoimmune reactions, immunological surveillance mechanisms and congenital immunological defects. Although the aim of these investigations has been predominantly oriented to elucidate the cellular processes involved in these phenomena, the ultimate aim is to find and study, using the model of *in vitro* human cell cultures, cell products with potential therapeutic use in diseases involving aberrant immunological reactions.

Part B

Publications:

1. Oppenheim, J. J. and Goldstein, G.: Enhanced thymic lymphocyte response to phytohemagglutinin in experimental autoimmune thymitis. Nature, 222: 192-193, 1969.

2. Oppenheim, J. J., Rogentine, G. N., and Terry, W. D.: The transformation of human lymphocytes by monkey antisera to human immunoglobulins. Immunol. 16: 123-138, 1969.

3. Oppenheim, J. J.: Immunological relevance of antigen and antigen-antibody complex stimulated lymphocyte transformation. Ann. Allergy. In press.

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: "Chromatography of Mammalian RNA and the RNA of Mammalian Viruses."

Previous Serial Number: None

Principal Investigator: Dr. R. Stern

Other Investigators: None.

Cooperating Units: Dr. R. M. Friedman (NCI), Dr. H. B. Levy (NIAID).

Man Years:

Total:	2
Professional:	1
Other:	1

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.

Project Description:

Objectives:

1. Whenever viral components have been sought in naturally occurring malignant neoplasms, only RNA viruses have been isolated. This suggests that RNA rather than DNA viruses may be the cause of human cancer. The biology of human cancer differs from that of other animals. No mature virion is ever detected in the fluid from human tumor cells grown in tissue culture. Our objective is to detect intermediates in the replicative cycle of RNA viruses in human tumor cells, rather than look for mature completed virus.

2. In order to look for RNA virus intermediates, it was necessary to recognize all the classes of RNA which occur in the normal mammalina cell. Mammalina RNAs which have been described to date are primarily those which also occur in bacterial cells, e. g. transfer and ribosomal RNAs etc. It can be estimated that the mammalina cell has one order of magnitude more classes of RNA than the bacterial cell, many of which have not been described. Another objective, then, has been the refinement of techniques for the separation, purification, preparation and analysis of all animal cell RNAs by column chromatography. We have observed in the course of our work and are describing a hitherto undiscovered form of RNA in animal cells which appears to share its biosynthetic mechanism with RNA viruses. Its possible relevance to the question of the viral etiology of neoplasia and abnormal cell proliferation are being examined.

3. In order to understand the replicative cycle of RNA viruses, we are developing techniques for the separation of the intermediates of a known RNA virus, the Semliki Forest Virus, a Group A Arbovirus, which has a very small RNA genome.

4. One of the criteria for viral infection in mammalina cells is response to interferon and inducers of interferon. We are looking for responses of RNA species in mammalian cells to interferon; normal, neoplatic, and cells infected with a known virus.

Methods:

Cell lines which are being maintained in primary or continuous tissue culture:

1. resting peripheral lymphocytes (no virus)	slow growing normal cells, (a negative control)
2. PHA stimulated peripheral lymphocytes (no virus)	rapidly growing normal cells, (a negative control)
3. Burkitt lymphoma cells (lines Al, Al_2, Al_3)	rapidly growing abnormal cells, (unknown cell line in which the putative RNA virus and causitive agent in human leukemia is being sought)
4. Murine leukemia virus infected mouse cells (MS-1) a lymphocyte line which has been malignantly transformed to leukemic state by a known RNA mouse leukemia virus	rapidly growing abnormal cells, (a positive control)

5. Chick fibroblasts infected with Semiliki Forest Virus (infected with a known RNA virus producing a lytic infection) rapidly growing abnormal cells, (positive control)

Lymphocytes are maintained in continuous culture (Burkitt lymphoma cells, Maloney murine sarcoma virus transformed cells) or grown in primary cultures (resting and phytohemagluttinin stimulated cells purified on a nylon column from a normal human donor), or virus is used to infect chick embryo fibroblasts. Cells are labeled and RNA extracted with phenol, detergents, and precipitated. These methods are being marked out step by step in an attempt to recover by a reproducible method viral-like RNA. Further purification, characterization, and resolution is done by column chromatography. Some of these techniques are being worked out in this laboratory or are established techniques from bacteriophage work being used for the first time on mammalina cells and mammalina RNA viruses. Some of these chromatographic techniques include benzoylated-DEAE cellulose, methylated albumin-sililic acid, polyacaylamide and polystyrene columns.

Major Findings:

A. Characterization of chromatographic properties of RNA using a new type of column resin, benzoylated DEAE cellulose (BD-cellulose). This column has never been used for mammalian RNA or for any RNA other than transfer RNA. Column properties were evaluated using synthetic RNA homopolymers, of known size and structure, copolymers, polymer duplexes and triple stranded polymers, plus isolated RNAs from physiological sources of known secondary and tertiary strucutres. Gradients were worked out by trial and error so that finally 90% of the total RNA of the mammalian cell was eluted in a single column run. Simple linear salt gradients elute RNA on the basis of secondary structure alone, and elutes RNAs which have a high degree of secondary structure. Elution is independent of size. Urea and/or DMSO plus an acidic pH are needed to elute single-stranded RNAs (except pC). Completely base paired double stranded RNA is the first RNA specie to be eluted. This RNA was thought to occur only in RNA viral infected cells. Double stranded RNA has been found in unifected cells.

B. Characterization of intermediates of replicative cycle of an RNA virus on the BD-cellulose column. Semliki Forest Virus RNA in chick fibroblasts was used to obtain labeled intermediates. Actinomycin pretreatment prevents synthesis of host RNA in this system.

Replicative form (RF) is the first RNA to be eluted from the column (entirely double stranded) followed by various replicative intermediates (RIs). RI elution progresses sequentially as the proportion of double strandedness decreased (as size of nascent single stranded RNA attached to RF template increases in length). The single stranded RNAs, including infectious RNA found in the mature virion, are entirely separated from the RI and RF, being eluted in a DMSO and urea gradient.

C. Attempt to find viral-like RNA in Burkitt lymphoma cells. Can viral-like RNA be detected in Burkitt lymphoma cells treated with actinomycin? Though actinomycin prevents synthesis of mature virion in other tumor systems, such as the Rous-sarcoma virus system, evidence for synthesis of viral-like RNA can be detected. Can similar RNA be found in the Burkitt cells? By combinations of treatment with cycloheximide, actinomycin, interferon, interferon inducers such as pIC, removal of ribonucleases by Sephadex G-100 chromatography, extraction in DMSO and by comparison with control cells such as the 48 hr. PHA stimulated lymphocyte, it is hoped to detect and characterize such an RNA species.

After 48 hrs. the PHA stimulated lymphocyte incorporates $^{3}H(U)$ into RNA at 80% the level of the Burkitt cell. This makes it an excellent control cell line for such experiments. Another cell line which has been used as a control is the murine leukemia cell line. These cells continually generate virus into the medium. Detection of intracellular viral RNA synthesis in this cell line in which virus is known to be actively synthesized will be used as the criterion of success in extractions.

Results to date have been varied. The viral RNA is highly labile. Extraction techniques are being worked out with the positive control cells. Temperature, Mg^{++} concentration, detergents, proteolytic treatments to remove ribonuclease activity, column techniques to separate RNA from the ribonuclease activities are variables which are being examined, one at a time.

D. Characterization of a new 4-7S RNA species found in all mammalian cell lines which have been examined to date with a s.8s RNAase resistant double stranded core, synthesized in the nucleus, appearing in cytoplasm after 25 minutes. This RNA is unmethylated and synthesized in the presence of very high doses of actinomycin. It represents 1% of the RNA synthesized in the cell in the absence of actinomycin.

It has also been found in rat liver, spleen, and kidney after in vivo actinomycin treatment.

E. Characterization of the total RNA from mammalian cells. RNA has been extracted from lymphocytes, both resting and PHA stimulated from Burkitt cells and from ^{3}H-orotic acid labeled rat liver. RNA was placed on sucrose gradients and then on BD-cellulose column.

The single 4-5s RNA peak from sucrose gradients contains 6 to 8 RNA species, all emerging in NaCl gradient, when examined on BD-cellulose. Only 3 have been described to date in the literature.

The 18s ribosomal RNA is one species of RNA which is eluted by DMSO.

The 28s rRNA contains 4-5 species, 3 emerging in the NaCl gradient. This heterogeneity of ribosomal RNA is undescribed to date.

Significance to Dental Research:

The search for an RNA virus as the causitive agent in human cancer is of obvious importance. Specifically to the dental profession, the Burkitt lymphoma is of immediate relevance.

It is an infiltrative lymphoma of bone in African children, usually seen initially by the dentist, since affected children present themselves with a maxillo-mandibular mass. The diagnosis of Burkitt lymphoma, which is closely related to acute lymphocytic leukemia, is becoming increasingly prevalent in North American children, as the level of awareness rises in the medical and dental profession. Indeed, a number of cases in Caucasian children have been studied at the NIH, and a number of cell lines in continuous culture derived from these patients, in addition to the lines from African children, are being used in this laboratory. Of all neoplastic processes, the epidemiology of the Burkitt lymphoma is the most suggestive of an infectious, and probably viral process. The search for an RNA virus in the Burkitt lymphoma line, or evidence for intracellular intermediates in the replication of such a virus are of obvious importance.

Proposed Course of Project:

We propose to continue searching for the RNA virus which causes the Burkitt lymphoma, for intermediates in the replicative cycle of the virus in the Burkitt and Maloney tumor cells (to date no intermediates have ever been detected by others in an RNA tumor viruses such as the Rouse Sarcoma virus, or Maloney virus in mammalian cells).

A by-product of this work will be the elucidation and characterization of many of theother classes of RNA molecules found in the normal mammalian cell.

Part B

Publications:

1. Baron, S., Bogomolova, N. N., Billiau, A., Levy, H. B., Buckler, C. E., Stern, R., and Naylor, R.: Induction of interferon by preparations of synthetic single stranded RNA. Proceedings of the Internationsl Symposium on Interferon, Lyons, France, January 6-9, 1969. In press.

2. Baron, S., Bogomolova, N. N., Billiau, A., Levy, H. B., Buckler, C. E., Stern, R., and Naylor, R.: Induction of interferon by preparations of synthethic single-stranded RNA. Submitted to Proceedings of the National Academy of Sciences, Washington, D. C.

3. Stern, R., and Friedman, R.: Resolution of the intermediates of an RNA virus on columns of Benzoylated-DEAE cellulose, Virology. In press.

Report of the Acting Clinical Director
National Institute of Dental Research

Fiscal Year 1969

The retirement of the former Clinical Director and Chief of the Oral Medicine and Surgery Branch during fiscal year 1969, coupled with the loss of a few other clinical personnel, the Government-wide restrictions on personnel hiring and budget, and the impending retirement of the Chief of the Dental Services Branch, has prompted a reappraisal of clinical programs within the Dental Institute. This reappraisal period is not viewed as an unwelcome development; rather it affords an opportunity to take a critical look at our role in the coming years within the boundaries of proper research philosophy and of the changing moods of Government and populace.

The goal of the clinical programs is to determine those methods whereby dental health can be maintained at an optimal level throughout a person's life. This, of course, is a direct and straightforward statement of obvious purpose. As such, it should not even need saying. Yet, there are times when some of us become so engrossed in individual pursuits that we do indeed need to be reminded of that simple statement of purpose.

To approach our goal, we need to become more familiar with programs outside our immediate area. For example, greater emphasis should in future be placed upon clinical application of the findings of our colleagues in basic research. Toward this end several moves are being considered: (1) Greater interaction between the clinical area and the basic research area will hopefully be fostered by scheduling time for the clinicians to work in basic laboratories with basic scientists on projects of mutual interest. This is something that has been done in the past on a very tenuous piecemeal basis, but beginning July 1, identifiable time will be allotted each clinician in the Dental Services Branch for the specific purpose of becoming involved in research and the learning of research techniques. (2) A new NIDR Branch devoted to Experimental Pathology has recently been created. It may serve the very useful secondary purpose of bridging the gap between clinical programs and basic research. (3) Cooperative and collaborative programs with other Institutes, principally with the Institute of Arthritis and Metabolic Diseases, the Institute of Child Health and Human Development, and the Cancer Institute, are being developed and/or extended with the expectation that mutual benefit will accrue through new approaches to common problems.

It has been predicted that dental caries will be eliminated possibly in another decade. Following the elimination of dental caries, it is conceivable that periodontal disease can eventually be significantly diminished. Despite these possibilities, however, the Dental Institute has a broadening stake in the future. The focus of dental research has been gradually enlarging to the point where few persons in the field now regard dentistry as being the art of filling teeth and treating gingival inflammation. Today, dentistry is generally regarded as that division of the health profession which deals with diseases, systemic as well as local, that may affect the oral cavity. This, then, demands that dental clinical research embody a variety of disciplines. Several examples can be pointed out.

Tooth implantation, replantation, and transplantation is an area of dentistry that has received scant attention in the past but will receive more concentrated attention in the very near future. This area of dental research endeavor will necessarily involve any number of facts such as immunology and graft or organ rejection phenomena, tissue healing, surgical technique, physiology of hard tissues, materials technology, carcinogenesis and toxicity, and biomechanical engineering.

The study of Behcet's syndrome, a syndrome with oral manifestations, points out rather clearly that we must understand extraoral disease in order to hope to effectively treat the intra-oral component. It may well be that the studies being carried out by the Dental Institute will have implications for other connective tissue diseases.

Disturbances of the temporomandibular joint have been greatly neglected until now. Reasons for this neglect include a reticence to deal with the joint for cosmetic reasons and its relative inaccessibility for thorough in vivo study. A study of arthritides of the temporomandibular joint with collaboration from other National Institutes of Health components is now in the planning stage.

Studies during the past ten years have yielded good baseline data on the use of general anesthesia for ambulatory dental patients. These studies have also suggested, however, that some undesirable physiologic alterations may occur while the patient is undergoing anesthesia. The studies are being expanded to follow-up on these findings and it may be that they will have a significant impact on the use of general anesthesia for ambulatory dental patients.

While studies of abnormality are of undeniable significance, the determination of what is normal is of at least equal, if not greater, importance. During the year, Dental Institute investigators have contributed much to the understanding of normality of the oral environment--in taste and oral sensory perception, in oral and pharyngeal development,

in salivary composition, and even in development of the rat skull. In collaboration with the Institute of Child Health and Human Development and with the Naval Hospital, a study of postnatal oral development in the human infant is being initiated. By developing this background fund of knowledge relating to normal development, we will be placing ourselves in a much better position to understand the abnormalities that may develop.

In summary, the year has been one of planning for a transition of emphasis. This statement reflects the thinking that has gone on during the year about that transition more than being a statement of definitive activities during the year. Dental diseases of a strictly localized oral nature will most assuredly not be ignored. Studies, for example, of caries etiology, prevention, and therapy of the causes and treatment of periodontal disease, of pulpal reaction to various mechanical procedures and restorative materials, will be continued. Some of the purely service aspects of the clinical programs will probably be diminished without cutting back on our service obligation to Clinical Center inpatients. But our thinking about the clinical programs area of the Dental Institute is being re-oriented toward taking a broader view of dental disease in the future and being an integral part of the study of dental disease as it affects well-being of the total individual.

Report of the Oral Medicine and Surgery Branch
National Institute of Dental Research

Summary Statement
Fiscal Year 1969

The Chief, Oral Medicine and Surgery Branch, retired October 1, 1968. The following is a summary statement of dental clinical projects carried out during the fiscal year.

A. Dental Diseases

1. Periodontal disease: The long-term study of periodontal disease among volunteers from the District of Columbia Fire Department designed to obtain baseline data on clinical signs of periodontal disease and to follow a stable population through a period of time is continuing. The initial examinations are now completed and statistical analysis of the gathered data is in process.

Clinical studies of periodontal disease among children and adolescents have provided evidence for stress functioning as an etiologic agent. In some instances the condition appears to be either initiated or at least aggravated and perpetuated by self-mutilation on the part of the child. In collaboration with a psychiatrist who interviewed the patients and their parents, it was hypothesized that gingival mutilation was used by the child as a means of coping with anxiety due to dependency conflicts.

Continuing animal studies of calculus formation showed this year that at least some factors involved may be transmissible, although the method of transmission has not been identified. A calculus-resistant strain of rats, when housed with a highly susceptible strain, showed significantly increased amounts of calculus deposition. It was also shown that incorporation of penicillin in the diet would reduce calculus formation, but it was also shown that diet could control calculus formation to an even greater degree. These confusing findings and observations point out strongly the need for carefully controlled studies of calculus formation.

A study of dilantin gingival hyperplasia was initiated this year. Animals generally do not develop gingival hyperplasia due to dilantin ingestion, although in humans it probably develops in more than half of those taking the drug. One group has, however, reportedly been

successful in inducing dilantin gingival hyperplasia in cats. The study here will attempt to induce hyperplasia in cats and will also use biopsy material from humans ingesting dilantin for the treatment of epilepsy. Histologic, histochemical, and enzymatic analyses will be made in an attempt to determine the causes of the hyperplasia.

2. Caries: Studies of dietary effects on caries were this year concentrated on an evaluation of the anticariogenic effects of fish protein concentrate. This concentrate has a high fluoride content and does appear to be a relatively noncariogenic food in rats, presumably due to the fluoride present.

   In a study of Caucasian and Negro males of similar age in the same community, it was found that the Caucasians had experienced twice as much caries as the Negroes despite the fact that all were supplied by the same water supply containing one part per million of fluoride and the Negro boys reported eating sweet foods more often and, inafact, eating more often than did the Caucasian boys. Specific caries-inducing streptococci were recovered from approximately twice as many cultures of plaque removed from Caucasian boys as well. The reason for these apparently contradictory findings are not yet known, but the study will be continued by analyzing enamel biopsies and studying fluoride excretion patterns.

3. Pulp studies: The effect of steroid preparations on pulp healing and pulpal response to cavity preparation was further evaluated. Although the steroids and obtundents had no effect on healing of exposed pulps in germfree rats, they did appear to limit or minimize the pulpal response to cavity preparation in humans.

B. Anesthesiology and Oral Surgery

1. Premedication: Since the majority of oral surgical procedures are performed in the dental office under local anesthesia, premedication is an important consideration. A study has been initiated to evaluate the suitability of various premedicating drugs for this purpose. This study involves the use of at least four different drugs on four separate occasions in the same patient and monitoring of vital signs before, during, and following the surgical procedure. This will allow comparison of drug effect within as well as between study subjects and will eventually allow for an assessment of the desirable characteristics of each drug versus the adverse characteristics.

2. General anesthesia: With what appears to be increasing acceptance of general anesthesia, particularly among oral surgeons, for use in the dental office on ambulatory patients, it is becoming a matter

of necessity that reliable data be gathered to define what is happening to the patient under these conditions. Fortunately, the National Institute of Dental Research has been studying the effects of general anesthesia on ambulatory dental patients for several years. These continuing studies have provided some rather enlightening findings and have, in addition, now progressed to the point of serving as a reliable baseline for evaluation of new anesthetic agents that may be developed. Significant elevations in blood pressure and pulse rate, exceeding what would be acceptable in a modern hospital operating room, are found consistently in dental ambulatory general anesthesia. Strong evidence suggests that the hypertension is related to surgical trauma and that the pulse rise is drug related. Furthermore, the incidence of cardiac arrhythmias in dental ambulatory patients under general anesthesia has been established to be within the range of 60 to 75 percent, as contrasted to the 30 to 35 percent reported incidence in general surgery. These findings may have a significant impact upon the use of general anesthesia in a dental office, particularly in those cases in which the patient has some degree of cardiovascular compromise. The studies will be continued on an expanded basis to determine just how serious these findings may be. Liver function tests that have been instituted as a part of the study have as yet been essentially negative.

3. Tissue healing: A study of the development of postoperative localized osteitis and its response to medication has been initiated. The use of medicaments after the removal of an impacted third molar on one side has not as yet shown any detectable difference in the subsequent development of osteitis as compared to the use of no medicament following extraction on the opposite side. A study of the effects of bilateral osteotomy, for the establishment of a new occlusion, upon the temporomandibular joint is continuing.

4. Tooth implantation: A study of tooth implantation conducted in a baboon population emphasized the importance of retaining as much of the cementum and periodontal fibers as possible on the extracted tooth during the implantation procedure and retaining this material in as viable a condition as possible by maintaining it in a moist environment during manipulation extra-orally. There is a suggestion that viability may be too seriously compromised if the extra-oral endodontic procedures exceed one-half hour. The portions of teeth from which cementum was removed were rapidly subjected to resorption.

C. Oral Ulcerations and Oral Manifestations of Systemic Diseases

1. Aphthous ulcerations and aphthous stomatitis, most often regarded as an annoying nuisance, are occasionally of such severity as to be life threatening. In continuing the long-term study of these

lesions, a concerted effort has been directed toward establishing an etiology. It has been determined that the frequency and severity of aphthous ulcers is a completely random phenomenon having no correlation with any clinical variable yet identified. This is in contrast to the hypotheses of some investigators who have attempted to show a relation between aphthous ulcerations and menstrual cycle or stress. A case for the establishment of Streptococcus sanguis strain 2A as the etiologic agent is being made in in vitro and cell culture studies. Progress in developing a therapeutic approach to the disease has been minimal. In a single case involving a severely affected patient, immunosuppressive drug therapy resulted in a protracted period of remission. Studies directed toward the definitive identification of an etiologic agent and an effective therapeutic regimen are continuing at the Bethesda campus and at the Dental Institute supported San Francisco facility.

2. Behcet's syndrome: The study of Behcet's syndrome, which may carry a mortality of more than 10 percent and which can result in lingering pain, blindness and neurologic disability, is now in its second year. The principal initial effort has been in defining the natural history of the syndrome, but gradual entry into a determination of the basis of the disease is now being made. An etiologic agent, if one exists, has not yet been defined. There has, however, been some interesting observations. It has been shown that most of these patients exhibit a moderate elevation in alpha-2-globulins and that some have elevated titers of cold agglutinins. Even in those patients who have no clinically or electroencephalographically demonstrable evidence of neurologic disease, it has been observed that spinal fluid proteins may be elevated. Many patients do have emotional problems which often border on psychosis and it is frequently possible to correlate the clinical onset of oral ulcers with psychologically significant occurrences. Antigens prepared from human uvea have been shown to stimulate precipitating antibodies,in animal experimentation, but antibody activity has not been demonstrated in the sera from patients with Behcet's syndrome. The development of a therapeutic program has not yet been seriously considered. It has, however, been observed that the oral ulcers of Behcet's syndrome seem to respond, much as aphthous ulcers do, to local and systemic tetracycline therapy. These studies are to be continued and expanded in conjunction with studies of other connective tissue diseases and with collaboration with other institutes.

One patient with Behcet's syndrome had serum positive for cryoglobulins. A study related in a minor way to this was related to the mechanisms of cryoprecipitation in cryoglobulins. In this study the relationship between protein solubility and structure with regard to temperature was evaluated thereby gaining further insight into the immunologic behavior of proteins.

3. Systemic diseases: A clinicopathologic correlation of a small series of cases of North American blastomycosis with oral lesions was completed during the year and prepared for publication. The study of dental pulpal involvement in leprosy was continued on teeth collected from two Public Health Service facilities and from the leprosarium at Cebu City in the Phillipines. These studies are intended only to point out the oral involvement that may be seen in some of the unusual fungal and bacterial diseases.

D. Histopathology of Oral Mucous Membranes and Oral Carcinogenesis

Two separate studies during the year were directed toward a redefinition of our understanding of the epithelial condition termed leukoedema. One was concerned with patients at the Clinical Center and the other with Bolivian Indians who apparently have a high incidence of leukoedema due to their habit of chewing Coca leaf. Both studies defined the condition but failed to demonstrate any evidence that leukoedema represents a premalignant lesion as it had been surmised to be in the past by some workers.

A previously undescribed change noted in the superficial epithelial layers of biopsy specimens from chronic hyperplastic oral lesions was determined to represent an epithelial product possibly related to an environmental agent such as denture material.

Submucous fibrosis, a possibly premalignant lesion, was studied in hamsters. A component of chili powder commonly used in India where the lesion is common has been suspected as a possible etiologic agent. Hamsters that have had this particular chili powder applied to their cheek poiches for 12 months have shown an unusual staining reaction in the superficial collagenous connective tissue and have just begun to show initial histologic characteristics of submucous fibrosis.

The attempted induction of squamous cell carcinoma in hamsters and baboons by use of betel quid and other suspected carcinogenic materials is continuing. After 12 months the baboons are showing hyperkeratinization and cellular atypia but no frank carcinoma has yet been detected. An interesting sidelight of this study is that the animals on betel have marked periodontal disease.

The study of fibro-osseous lesions that involve the jaws was continued with a specific study of cherubism and its treatment. The perivascular eosinophilic material that has been noted previously by many independent investigators in biopsy material from cherubism was histochemically identified as collagen. This should serve as a valuable aid in differential diagnosis of this particular lesion among the other fibro-osseous lesions that may appear histologically similar. Treatment of patients with cherubism is probably dependent upon thorough evaluation of the individual case, with treatment recommendations varying with the age of the patient and aggressiveness of the lesion, but with thorough curettement being the treatment of choice when possible.

Statistical analysis of a series of chondrosarcomas involving the jaws was completed during the year. These tumors appear to behave somewhat more aggressively in the jaws than in other bones and to have a slightly worse prognosis.

E. Neuromuscular Function and Development

Studies of taste and oral sensory perception this year led to better localization of taste receptors. Receptors were specifically defined on the palate and tongue and recognition of the four basic taste sensations was recorded.

Comprehensive study of five children with deficiency of taste or smell and who also had orofacial developmental problems suggested that there may be a relationship between the development of the special senses of taste and smell and malocclusion due to skeletal discrepancy. This aspect of these studies will be followed up.

Using displacement and pressure transducers, it was possible to study oral motor mechanisms. Lingual and palatal pressures during speech and swallow were recorded. These studies will be continued in order to determine the role of muscle pressure in the development of normal dentition and malocclusion.

The effect of muscle pressure upon condylar form following condylar injury was also a subject of study. This study has elicited evidence that the asymmetry that often follows condylar injury in preschool children may be largely preventable simply by compelling the child to use the muscles on the affected side more effectively. Now in its seventh year, this study has provided evidence also that the bony structure of the temporomandibular joint may be retained in a relatively normal pattern through judicious direction of muscular activity following condylar injury.

Further evidence that early orthodontic intervention in patients with palatal clefts is advantageous was gathered.

A study of laryngeal development using autoradiographic techniques in chick embryos showed an interesting migration of neural crest tissues and presented evidence that the laryngeal cartilages and connective tissues in the chick are of neural crest origin.

Serial No. NIDR-67 (c) (63)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Histopathology of Oral Mucous Membrane

Previous Serial Number: NIDR-65 (c)

Principal Investigator: Dr. H.O. Archard

Other Investigators: None

Cooperating Units: National Cancer Institute, Dermatology Branch

Man Years:

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

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 other pathologic mucosal tissue. On the basis of its morphology and the histochemical finding of -SH groups, it probably represents an epithelial product. It appears to be most prevalent 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 other clinical white lesions, some of which are of a premalignant or malignant nature.

2. The identification of the unusual changes 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. O. 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" (Accepted for publication).

3. Archard, H.O.: Verrucous carcinoma of the oral cavity. Transactions of the Third International Conference on Oral Surgery, J.P. Lippincott Co., Philadelphia (In press).

Serial No. NIDR-68 (c) (59)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Studies on the Etiology and Treatment of Periodontal Diseases in Children and Adolescents

Previous Serial Number: NIDR-67 (c)

Principal Investigator: Dr. P.N. Baer

Other Investigators: None

Cooperating Units: Dr. H. Hoffman, NIMH

Man Years:

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

Project Description:

Objectives:

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:

Children with bizarre gingival lesions which do not correspond to any known gingival disease or oral manifestations 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 received 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 are being pursued.

Part B

Publications:

1. Hoffman, H.A. and Baer, P.N.: Gingival mutilation in children. Psychiatry 31: 380-386, 1968.

Serial No. NIDR-69 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Studies on Experimental Calculus Formation

Previous Serial Number: NIDR-68 (c)

Principal Investigator: Dr. P.N. Baer

Other Investigators: Dr. P.H. Keyes, Mr. C.L. White, Mr. G.R. Hawkins

Cooperating Units: Mr. N. Mantel, National Cancer Institute

Man Years:

Total:	1 1/2
Professional:	1/2
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 rats 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 scored 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., 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. 39: 86-88, 1968.

2. Baer, P.N.: Use of laboratory animals for calculus studies. Proc. N.Y. Acad. Sciences 153: 230-239, 1968.

Serial No. NIDR-70 (c) (68)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: The Effects of Blastomycosis on Oral Tissue

Previous Serial Number: NIDR-69 (c)

Principal Investigator: Dr. W.A. Bell

Other Investigators: Dr. G.E. Garrington

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To define the gross appearance, histologic appearance, and radiographic appearance of oral lesions of North American blastomycosis.

Methods Employed:

The National Institutes of Health hospital records of patients with North American blastomycosis who had intra-oral lesions associated with the disease have been reviewed and additional follow-up material has been obtained. These records have been reviewed and histologic material from the oral lesions has been examined to determine the character of the lesions and the fate of the lesions following treatment.

Major Findings:

1. North American blastomycosis may produce intra-oral lesions involving both soft tissue and bone. When bone is involved, the disease may produce extensive destruction.

2. The intra-oral soft tissue lesions of North American blastomycosis are not grossly characteristic enough to enable definitive diagnosis without biopsy.

3. The intra-oral lesions of North American blastomycosis heal readily when the systemic disease is adequately treated with chemotherapeutic agents. Alveolar bone that has been lost as a result of the disease does not apparently regenerate.

Significance to Dental Research:

There are no reports in the literature of series of cases of North American blastomycosis with oral lesions. Such a report is desirable in order to better evaluate clinical behavior of the lesion and results of treatment.

Proposed Course of Project:

Collection and evaluation of patient materials is now completed. A manuscript reporting the findings of the study has been prepared and is now in the process of review for publication. The project will be terminated with publication of the paper.

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, 1968 through June 30, 1969

Part A

Project Title: A Measure of the Effectiveness of Root Amputation as a Means of Slowing Pocket Progression

Previous Serial Number: NIDR-71 (c)

Principal Investigator: Dr. A.F. Binderman

Other Investigators: None

Cooperating Units: None

Man Years:

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

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 the 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, the limited size of the group of teeth studied seems to show a trend toward more effective maintenance in the root amputated tooth.

2. Patients, when questioned about difficulty in gaining access to these areas, do feel that they themselves are more effective in the curetted areas.

3. It has been a difficult task to obtain a smooth surface of tooth after a root amputation due, in large part, to the mechanism of root removal.

4. Thus far, not enough information is available to determine the rate of change in pocket depth within the root-amputated area of the tooth.

Significance to Dental Research:

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 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.

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, 1968 through June 30, 1969

Part A

Project Title: Studies in the Healing of Alveolar Bone in Dogs

Previous Serial Number: NIDR-72 (c)

Principal Investigator: Dr. A.F. Binderman

Other Investigators: Dr. P.M. Lightbody and Dr. P.N. Baer

Cooperating Units: None

Man Years:

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

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 under 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 predictably 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, decorticalication 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, 1968 through June 30, 1969

Part A

Project Title: Study of Behcet's Syndrome

Previous Serial Number NIDR-73 (c)

Principal Investigator: Dr. N. A. Cummings

Other Investigators: Dr. T. C. Francis, Dr. D. R. Bergsma

Cooperating Units: NIAMD, Arthritis and Rheumatism Branch; NINDB, Ophthalmology Branch; NINDB, Medical Neurology Branch; NIMH, Laboratory of Clinical Science; NCI, Dermatology Branch

Man Years:

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

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.

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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 sacroiliac joints is carried out.

6. Other clinical and laboratory parameters as indicated are obtained when necessary.

Major Findings:

1. No specific etiologic agent has been so far recovered. Oral, venous, and spinal fluid sources are negative for virus, PPLO, bacteria or fungi. Non-specific manifestations of inflammation, such as erythrocyte sedimentation rate, are often elevated. Two cases had elevated titers of cold agglutinins, and one had serum positive for cryoglobulins. The most constant serum finding is a moderate elevation of alpha-2-globulins.

2. Two cases, with no clinical or electroencephalographic evidence of neurologic disease, had moderate elevation of cerebral-spinal fluid proteins, one with a mild pleocytosis. Both of these patients had inflammatory disease of the eye.

3. Conjunctival biopsies show varying degrees of round-cell infiltration of blood vessels, but only where ocular inflammation was clinically detected.

4. One case of incomplete Behcet's Syndrome had retinal inflammation, with subsequent neovascularization and vitreous hemorrhage. It was only after several years' course that anterior inflammation, ostensibly the ocular hallmark of this syndrome, finally appeared.

5. Oral ulcers seem to respond to local and systemic tetracycline. However, patients generally do better soon after admission to the hospital setting.

6. Antigens derived from human uvea stimulate four precipitating antibodies in experimental animals. No antibody activity against

these absorbed antigenic eye proteins have been detected in sera of patients with Behcet's Syndrome by this system.

7. These patients have severe characterologic disorders, marked by orally-dependent and depressive personalities. They all tend to be emotionally immature, and some may border on psychosis. The onset of oral ulcers, in several cases, has been correlated in a startling manner with important psychological occurrences in their life. However, the character disorders are not thought to cause the Syndrome per se.

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 patients 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, 1968 through June 30, 1969

Part A

Project Title: Mechanisms of Cryoprecipitation in Cryoglobulins

Previous Serial Number: NIDR-74 (c)

Principal Investigator: Dr. N. A. Cummings

Other Investigators: None

Cooperating Units: Houston Veterans 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 spectrophotometric and chemical techniques is determined.

5. Relationships between structure and solubility is considered in light of the above data, with regard 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 an euglobulin, and satisfies criteria of purity immunoelectrophoresis, cellulose acetate electrophoresis, gel filtration, and analytical ultracentrifugation. It has an $S_{20,w}$ 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 1.0 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 reduction 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.

7. Addition of varying amounts of non-cryo-IgG does not alter the kinetics of cryoprecipitation. The cryoglobulin light chains have kappa specificity. Mixing with aliquots of pooled IgG prior to cryoprecipitation fails to reveal anything but kappa chains in the subsequent cryoprecipitate. Thus preliminary evidence is that this is a monoclonal protein with no antibody activity to normal IgG.

8. Mild reduction shows that the cryoglobulin seems somewhat more resistant to mercaptoethanol than normal gamma globulin. Preliminary "fingerprints" of the light chains reveal common kappa tryptic peptides; there may be more variable tryptic peptides than from pooled IgG.

Significance to Dental Research:

This work studies the relationship between protein solubility and structure with regard 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 pathologic 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.

2. Cummings, N. A.: Decreased tyrosine/tryptophan ratio in a 6.6S cryoglobulin, as determined by a spectrophotometric technique. Biochem. Biophys. Res. Com. 33: 165, 1968.

Serial No. NIDR-75 (c) (58)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: General Anesthesia on Ambulatory Dental Patients

Previous Serial Number: NIDR-75 (c)

Principal Investigator: Dr. E.J. Driscoll

Other Investigators: Dr. P.M. Lightbody and Dr. R.D. Fiorucci

Cooperating Units: Dr. C.L. Hebert, Anesthesiology Department, Clinical Center; Dr. C. Battig, Biomedical Engineering and Instrumentation Branch, DRS

Man Years:

Total	:	4½
Professional	:	2½
Other	:	2

Project Description:

Objectives:

For the past eleven years studies have been conducted by an NIDR-NIH research team on all the readily measurable physiological parameters of patients undergoing general anesthesia for dental surgery on an ambulatory basis.

Historically, the procedures of oral surgery have been closely identified with the administration of general anesthesia on ambulatory patients. Patient preference has always been highly weighted in favor of general anesthesia whenever it was available. This choice of anesthesia has not always been shared by oral surgeons because of an inadequate background of knowledge regarding the physiologic behavior of patients undergoing general anesthesia on an ambulatory basis.

There is a vast amount of scientific information on the hospitalized patient undergoing general anesthesia, but the results of the studies are not always applicable or interpolable.

Dental anesthesia differs from ordinary surgical anesthesia in several important respects. Among the more important are that the

patients are ambulatory and not usually sedated, and they expect to be incapacitated only for short periods of time; the operations are performed in and about the airway and in as light a plane as possible which factors introduce specific problems related to this type of anesthesia.

Because of these major differences, the background of knowledge in general dental anesthesia is incomplete. Thus a great need exists for fundamental data concerning alterations in the physiological mechanisms and a definition of the limits beyond which hazards are faced.

The accumulated data from this study have been and will continue to be used as a baseline of comparison for the many new anesthetic drugs which have been and are being proposed for use in oral surgery.

Methods Employed:

1. Full mouth extractions are selected rather than random oral surgical procedures so that the surgical procedure can be performed on a more predictable pattern and in order to standardize the surgical trauma. The teeth are extracted in quadrants, and the entire operation is systematized for overall time of procedure and traumatic experience. A four-channel polygraph is employed, which is electronically timed to deliver two feet of graph per minute. A staff member records all the anesthetic and operative data, accurately timed in seconds, on a separate data sheet and later transcribes this information directly on to the polygraph record. A "second" accurate, chronological narrative is thereby provided on the graph for the purpose of evaluating the time related surgical and anesthetic events, such as the effect of surgical stimulation on pulse and blood pressure.

2. 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:

One hundred and fifty detailed studies on an additional 47 adult patients are added for this reporting period, making an overall total of 1,300 operations performed. The operations average 25 minutes in length which is many times longer than the average

dental anesthetic employed in outpatient oral surgery, the longer time having been selected for previously outlined reasons.

Major Findings:

1. The EEG has continued to be of value in providing a reliable objective gauge of the depth of anesthesia during the procedure. The importance of this factor is realized when physiologic measurements such as blood pressure, pulse and O_2 saturations are made and studied in relation to the prevailing plane of anesthesia.

2. The respirometer provides a complete account of the patients' respiratory pattern ranging from the extremes of apnea to hyperpnea.

3. The Waters Earpiece Oximeter supplies a rather accurate ($\pm$ 3-5%) estimate of the patients' arterial O_2 saturation and relates intimately to respiratory activity or lack, thereof.

4. (a) A significant blood pressure elevation and pulse acceleration are consistently found in this type of ambulatory anesthesia. For the exact same operation performed in the operating room under hospital-type sedation and conditions, one does not find or would one tolerate such pulse and blood pressure responses. This is an extremely important practical finding, which the conscientious oral surgeon must take into consideration when accepting a patient with a compromised cardiovascular system.

   (b) It was found that the blood pressure and pulse elevation with methohexital, the present barbiturate under study, was slightly higher than with the standard reference drug Pentothal. The pattern of rise, however, is identical, i.e., the pulse peaks in one to two minutes, whereas the blood pressure reaches its highest point in six minutes, indicating a different mechanism of operation in these closely allied physiologic measurements.

   (c) The previously reported hypertension was principally of the systolic variety. We are now reporting a significant elevation in the diastolic system. The diastolic rise is probably related to the inability of the vascular system to obtain any measure of relaxation because of the rapidly beating heart associated with this particular intravenous barbiturate, methohexital.

   (d) We have enough data to say with certainty that the operative hypertension is related to surgical trauma, and its peak parallels that of the intensity and duration of the stimulation. The pulse activity gives strong evidence of having a drug (barbiturate) relationship. It coincides exactly with the drug response as shown on the EEG not only on the initial dose but on the many incremental administrations.

5. Cardiac arrythmias have been noted as an item of clinical interest from the time of our very earliest EKG recording over ten years ago. For this reason a special study has been instituted during the past year. An additional, continuous electrocardiograph is made on each patient utilizing a separate and more sensitive instrument, and the records are studied in relation to all the other recorded data. An astonishing incidence of arrythmias is being found to be in the range of 60-75%, as contrasted with a 30-35% reported incidence in general surgery.

6. A preliminary analysis of our five year liver function study of patients who have undergone Halothane anesthesia, is providing essentially negative results. These negative (no hepatotoxity demonstrated) findings are especially important since most of our patients have had at least two consecutive Halothane administrations. The repeated Halothane anesthesia were (and still are) slightly suspect in the alleged Halothane "toxicity" indictment. A more detailed and thorough study of the pre-anesthetic baselines and the post-anesthetic liver function findings should be of interest not only of Halothane but also of all the inhalation and intravenous anesthetics administered during the past five years.

Significance to Dental Research:

1. 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 acquired baseline data of physiologic response, it would be difficult, if not impossible, to evaluate them.

2. The possibility for the improvement and refinement of present technics of anesthesia is under study as well as the establishment of a 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.

3. Not only are the baseline which we have and are continuing to establish important to our general anesthesia research, but they will also be of equal importance to the study of local anesthesia supplementation with the various drugs presently being used and proposed for intravenous sedation and amnesia. Since these drugs are administered intravenously and are pharmacologically similar to the agents we have been studying, the proposed investigation will fit in well with our past studies.

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:

1. 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.

2. The incidence of cardiac arrythmias will continue to be studied in all types of ambulatory anesthesia. The situations under which arrythmias occur as well as other clinically important factors will be investigated and reported.

3. The technical means for determining the all important pO_2 and pCO_2 by means other than arterial puncture (not feasible in ambulatory studies) are now available. These methods will be explored when the necessary instruments are acquired and our support personnel are properly oriented and trained.

4. All the knowledge, experience and data which have been acquired in over ten years of physiologic studies in general anesthesia will be employed in our local anesthesia-premedication studies, which we are planning to undertake in the next few months.

Part B

Publications:

Research in anesthesia for ambulatory patients - practical considerations. Driscoll, E.J., Hebert, C.L. and Battig, C.G., Proceedings of the 3rd International Conference on Oral Surgery, J.B. Lippincott, Philadelphia, Pa., 1969.

Honors and Awards Relating to this Project:

Honorary Membership - Society for the Advancement of Dentistry in Anaesthesia, London, England, 1969.

Serial No. NIDR 76 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Tissue Culture and Microbiological Studies in Recurrent Aphthous Stomatitis, Behcet's Disease, Rheumatic Heart Disease, and Normals

Previous Serial Number: NIDR-76 (c)

Principal Investigator: Dr. T. C. Francis

Other Investigators: Dr. R. W. Lyman, Dr. J. F. Bosma, Dr. E. A. Graykowski, Dr. J. J. Oppenheim

Cooperating Units: Division of Biologics Standards, Dr. M. F. Barile

Man Years:

Total:	1 1/4
Professional:	1/4
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 pertinent diseases.

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 culture systems.

5. To investigate the role of the lymphocyte in host-defense mechanisms.

6. 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.

7. To collaborate with Dr. Cummings in the study of Behcet's Disease.

Methods Employed:

1. Lymphocyte transformation studies comprise the major emphasis of our work 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.

2. Routine patient care to maintain clinical patients.

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 20 patients comprise the study group.

Major Findings:

1. 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 occurrence, the number of lesions, and their severity is a completely random process and is not correlated to any other clinical variable recorded.

2. We have been able to show that a specific strain of organisms (*Str.* sanguis strain 2A) has the same effect *in vitro* on leukocyte cultures from patients with aphthous stomatitis that Lancefield group A strains have on *in vitro* leukocyte cultures from patients with rheumatic heart disease. We feel that this may represent the basis for this disease.

3. A more basic finding is that human pathogenic streptococci stimulate human cell cultures significantly more than non pathogenic strains and conversely, animal pathogenic streptococci stimulate animal lymphocytes significantly more than either non-pathogens or human pathogenic strains.

4. The treatment of a single aphthous patient with an immunosuppressive drug resulted in a period of remission from lesions.

5. There have been no significant developments on the patients with Behcet's Disease.

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, once again the emphasis being on tissue culture studies.

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. This part of the project should be the responsibility of a physician.

Beginning in September, the principal investigator, Dr. Francis, began a two-year period of training. Arrangements have been made to maintain routine follow-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. 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. 1968.

2. 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.). Published in 1969.

3. Francis, T. C., and Oppenheim, J. J.: Lymphocyte transformation studies to streptococcal antigens in patients with aphthous stomatitis, Behcet's disease, Rheumatic Heart diseases and Normals. J. Clin. Invest. (In press).

Serial No. NIDR-77 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Osteosarcoma and Chondrosarcoma of the Jaws

Previous Serial Number: NIDR-77 (c)

Principal Investigator: Dr. G.E. Garrington

Other Investigators: None

Cooperating Units: Armed Forces Institute of Pathology, Washington, D.C.

Man Years:

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

Project Description:

Objectives:

1. To establish improved criteria for diagnosis of these tumors in the jaws, thereby aiding in earlier diagnosis.

2. To evaluate therapeutic measures in terms of prognosis.

Methods Employed:

Case material, including medical history and operative data, roentgenographic studies, and histologic material as well as, current follow-up materials, was obtained for 92 cases of osteosarcoma or chondrosarcoma arising as a primary tumor in the jaws. This material was evaluated and correlated in order to establish patterns of clinical behavior, roentgenographic appearance, histologic character, therapeutic efficacy, and survival.

Major Findings:

1. As compared to the same tumors in other bones, osteosarcoma appears to have a slightly better prognosis and chondrosarcoma a worse prognosis. This is probably not due to any basic differences in the tumors themselves since they are histologically comparable to their counterparts in other bones. The difference in prognosis does appear, however, to be related principally to specific site of origin within the jaws and amenability to aggressive surgical attack.

2. Radical surgery offers the best hope of cure for either tumor.

3. An important early sign of the disease is a symmetrically widened periodontal membrane space. This finding, initially reported as a result of this study, has since been corroborated by Colonel William G. Sprague, Oral Pathologist, USAF, and by General Robert Shira, Oral Surgeon and Chief of the Dental Corps, U.S. Army, in a subjective review of the cases they have seen.

Significance to Dental Research:

1. This study has provided a better understanding of the characteristics of osteosarcoma and chondrosarcoma arising in the jaws, thereby enabling earlier diagnosis and potentially improving the survival rate.

2. This study has added to the already existent data indicating that there is an as yet poorly understood relationship between hormones and development and control of tumor growth.

Proposed Course of Project:

This specific project is essentially completed and will be terminated with the publication of a paper describing the chondrosarcoma portion. That paper is now near completion. A similar project directed toward study of the myxoma-myxosarcoma group of tumors as they occur in the jaws is being considered for the future.

Part B

Publications:

Materials from this study have been requested for use in the discussion of osteogenic tumors in the revised edition of Thoma's Oral Pathology, soon to be published.

Lectures:

The findings of this study have been the basis of lectures at:

a. The Armed Forces Institute of Pathology Course of Pathology of the Oral Regions, 1968 and 1969.

b. A seminar at the Medical College of South Carolina, February 1968.

c. The U.S. Naval Dental School Continuing Education Course in Oral Pathology, January 1969.

Serial No. NIDR-78 (c) (67)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Leprosy Involving the Dental Pulp

Previous Serial Number: NIDR-78 (c)

Principal Investigator: Dr. G.E. Garrington

Other Investigators: None

Cooperating Units: U.S.P.H.S. Hospital, Carville, Louisiana; U.S.P.H.S. Outpatient Clinic, San Pedro, California; U.S.P.H.S. Hospital, San Francisco, California; Leonard Wood Memorial (Cebu City, Phillipines, Leprosy Laboratory)

Man Years:

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

Project Description:

Objectives:

1. To determine the effects on the dental pulp and adjacent tissues of infection with Mycobacterium leprae.

2. To determine a rational approach to therapy for teeth involved in leprosy.

Methods Employed:

Teeth extracted from patients with leprosy have been collected from four sources: Public Health Service facilities at NIH, Carville, and San Pedro, and the Leprosy Laboratory at Cebu City, Phillipines. These teeth are being examined histologically and histochemically in both ground section and paraffin section to determine the effects of leprosy infection on pulp, tooth, and surrounding tissues.

Major Findings:

1. Mycobacterium leprae invade the dental pulp via systemic dissemination and lead to pulp degeneration and the production of dental symptoms.

2. Mycobacterium leprae invade dentinal tubules of intact caries-free teeth.

Significance to Dental Research:

The sequence of events following pulpal infection with Mycobacterium leprae is not presently known. This study will define these effects.

Proposed Course of the Project:

The teeth that have now been collected will be evaluated. The project will be terminated with publication of the resultant paper.

Part B

Publications:

1. Garrington, G.E. and Crump, M.C.: Pulp death in a patient with lepromatous leprosy, Oral Surg., Oral Med., and 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, 1968 through June 30, 1969

Part A

Project Title: Dilantin Gingival Hyperplasia

Previous Serial Number: NIDR-79 (c)

Principal Investigator: Dr. G.E. Garrington

Other Investigators: Dr. H.M. Fullmer

Cooperating Units: V.A. Hospital, Washington, D.C.; U.S. Army Institute of Dental Research

Man Years:

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

Project Description:

Objectives:

1. To determine the mechanism by which sodium diphenylhydantoin produces gingival hyperplasia.

2. To study the morphologic and developmental character of dilantin gingival hyperplasia in humans and in experimental animals.

3. To assay enzymatic activity in hyperplastic human gingival tissue resulting from dilantin ingestion and to compare it with activity in animals ingesting dilantin as well as in normal human and animal gingival tissue.

4. To determine whether there are microvascular changes in dilantin hypertrophied gingival tissue as compared to normal gingival tissue.

Methods Employed:

1. Tissue is currently being collected from epileptic patients being seen at the V.A. Hospital. Tissue is to be collected from patients on dilantin who have hyperplasia; patients on dilantin who have no clinical evidence of hyperplasia; and patients on drugs other than dilantin. Occasional specimens will be obtained in the NIDR dental clinic from epileptic patients. An additional few specimens will be obtained from the NIDR dental clinic from non-epileptic patients for use as controls. Excised tissues will be treated in three ways: a portion will be fixed in formalin for routine histology; a portion will be frozen for enzyme histochemistry; and a portion will be incubated for quantitative analysis of certain enzymes.

2. Dilantin will be administered to cats to induce gingival hyperplasia. The same histologic examination and enzyme assays performed on human tissues will be performed on gingival tissues from the cats.

3. Microcirculation of cat gingiva will be studied by means of silicone rubber injections with the objective of comparing microvascularity in dilantin hyperplastic tissue with that in normal tissue from cats.

Major Findings:

The project is now in the initial stages of tissue collection from humans and dilantin administration to cats. There are no findings as yet.

Significance to Dental Research:

1. The study is designed to add to the meager (essentially non-existent) knowledge concerning the mechanism whereby dilantin leads to gingival hyperplasia. This may conceivably serve as a step toward determining the mechanism for other soft tissue proliferations.

2. Enzymatic assays of the gingival specimens will add to the knowledge of enzymatic effects and responses in gingiva which is also rather meager currently. The assay, particularly, of collagenase activity may provide an insight into its possible relationship to periodontal disease generally.

Proposed Course of the Project:

The study, now in its initial stages, will be continued until approximately 50 human tissue specimens have been collected and evaluated. Tissue specimens from at least 10 experimental cats will also be evaluated. Results of these studies will be reported and any promising leads regarding connective tissue proliferations will be followed up in further studies.

Part B Not included

Serial No. NIDR-80 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Carcinogenesis

Previous Serial Number: NIDR-82 (c)

Principal Investigator: Dr. J.E. Hamner, III

Other Investigators: None

Cooperating Units: Southwest Foundation for Research and Education, San Antonio, Texas

Man Years:

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

Project Description:

Objectives:

1. To induce squamous cell carcinoma in the hamster and the baboon buccal mucosa, using various betel quids with and without tobacco.

2. To study the specific effects of calcium hydroxide, betel pepper leaf, areca nut, catachu, and tobacco on the buccal mucosa as carcinogens or co-carcinogens.

3. To relate these findings to basic carcinogenesis and to the high rate of oral cancer in India and Southeast Asia.

Methods Employed:

1. NIDR hamster studies: Eleven groups of animals were placed on various combinations of calcium hydroxide, Indian tobacco, carbon tetrachloride (to induce cirrhosis), and 0.5% D.M.B.A. Cheek pouch biopsies were taken periodically to study any histologic changes. To date no carcinoma has been induced, norexpected yet, since this is a time-consuming process.

2. Southwest Foundation primate studies: I am the Project Officer for a contract with the Southwest Foundation in San Antonio (#PH-43-67-1475) entitled "Betel Quid Carcinogenesis in the Baboon." Dr. O.M. Reed is the investigator at that institution carrying out the contract which has run from 6/28/67 to 6/27/69 and is due to be extended until June 1970. The estimated support for fiscal year 1969 was $22,000.

   Twelve baboons have been maintained on a protein deficient diet, have had a surgically created pouch made in the buccal mucosa, and have been treated with the standard betel quid from Bombay with and without tobacco.

Major Findings:

1. Cirrhosis was successfully created in the hamsters injected with carbon tetrachloride, and carcinomas were induced in the D.M.B.A. treated animals. The animals on tobacco and calcium hydroxide either alone or in combination have not yet developed tumors.

2. After twelve months the baboons show epithelial atypia in some of the cheek pouch biopsies in the betel treated animals, but no frank carcinoma yet. Periodontal disease is marked in the animals on betel.

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:

The hamster project using calcium hydroxide and tobacco should continue another year to obtain results desired- carcinogenesis using these natural carcinogens requires a long period of time, but should tell us more about the tumor-producing properties of both. The betel quid study on primates hopefully should induce carcinoma sometime this forthcoming year. Both projects will continue on their present courses.

Part B Not included.

Serial No. NIDR-81 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Oncology

Previous Serial Number: NIDR-83 (c)

Principal Investigator: Dr. J. E. Hamner, III

Other Investigators: None

Cooperating Units: National Cancer Institute; Tata Institute of Fundamental Research, Bombay, India

Man Years:

Total:	1/4
Professional:	1/4
Others:	0

Project Description:

Objectives:

1. To continue to study the fibro-osseous tumors of the jaws by collecting pertinent cases from the Clinical Center patients, forming a registry for such lesions and other neoplasms.

2. To study epithelial premalignant lesions in rural Indian populations via collaboration as the Project Officer for P.L. 480 Project 644322 with Dr. Mehta in Bombay, India.

3. To serve as one of the collecting centers for premalignant epithelial lesions for the World Health Organization (NIDR Section of Oncology).

4. To study cherubism: its cause, pathogenesis, treatment, and prognosis.

Methods Employed:

1. Case histories, radiographs, histologic material, etc., are being collected in an Oncology Registry in the Section of Oncology, Laboratory of Experimental Pathology, NIDR.

2. P.L. 480 Project 644322 has been renewed for another three years in India. Collaborative investigations are being carried out on the patients with submucous fibrosis and leukoedema (see separate Project Reports).

3. Cherubism Studies: (A) Lesion specimens for histochemical analysis were obtained from five boys and three girls, aged 14 months to six years, with a familial history, clinical stigmata, and radiographic evidence of cherubism. Six children were Caucasians and two were Negroes.

   Histologic specimens were stained by the following methods:

   I. Protein
      (A) For collagen protein - Van Gieson's stain
      (B) For noncollagenous protein -
         1. Tyrosine - Lillie-Glenner (diazalization + S-acid) stain
         2. Tryptophane - Glenner-Lillie stain
         3. Cystine - Lillie stain
      (C) For elastica - Orecein stain
      (D) For reticulum fibers - Wilder's stain

   II. Carbohydrate - Periodic acid-Schiff reaction

   III. Mucopolysaccharide
      (A) Rinehart's stain
      (B) Azure A, pH 2, pH 3.5, and pH 4.5

   IV. Amyloid - Crystal violet stain

4. Cherubism Studies: (B) An analysis of treatment was done on two siblings with cherubism who were Clinical Center patients; a 5-year follow-up was done.

Major Findings:

1. The peculiar eosinophilic perivascular material noted around small vessels in cherubism lesions was demonstrated by the Van Gieson and Rinehart methods to be collagen protein.

2. Every child suffering with cherubism must be evaluated as a separate individual case in determining the method of treatment. During the early years of rapid onset of the jaw lesions and extreme growth, we found thorough curettement, sparing the permanent teeth buds and cosmetic deformity, if possible, to be the treatment of choice. The tumor growth did continue in both children regardless of curettement or trimming procedures in the original sites of tumor and in later developing tumor areas in both jaws. After

five years tumor growth was controlled, but still present. Cosmetic, swallowing, speech, and respiratory improvement was great.

Significance to Dental Research:

The demonstration of perivascular cuffing of collagen around the small vessels in cherubism lesions serves as a histologic marker to aid in confirming a positive diagnosis of this genetic lesion. The detailed follow-up of a five-year surgical history on the two cherubism patients should serve as a good guide for surgeons who are confronted with this difficult patient management problem.

Proposed Course of Project:

1. Oncology Registry: this will continue to be enlarged over the years as new cases are added.

2. P.L. 480 Project #644322: this study will continue for another three years.

3. W.H.O. Collecting Center: this registry will continue to enlarge as new cases present.

4. Cherubism Studies: If possible, cherubism cases will be added to the present collection of 8 cases during my three-year stay at the Southwest Foundation in San Antonio. Continued follow-up will be conducted on the two siblings who were Clinical Center patients, collaborating with Dr. A. Ketcham. New admission of cherubism patients will be sought in the Clinical Center.

Part B

Publications:

1. Hamner, J. E., III, Scofield, H. H., and Cornyn, J.: Benign fibro-osseous jaw lesions of periodontal membrane origin: An analysis of 249 cases. Cancer 22: 861-878, October 1968.

2. Hamner, J. E., III, Lightbody, P. M., Ketcham, A. S., and Swerdlow, H.: Cemento-ossifying fibroma of the maxilla. O. Surg., O. Med., and O. Path. 26: 579-587, October 1968.

3. Hamner, J. E., III: The demonstration of perivascular collagen deposition in cherubism. O. Surg., O. Med., and O. Path. 27: 129-141, January 1969.

4. Hamner, J. E., III, and Ketcham, A. S.: Cherubism: An analysis of treatment. Cancer (In press), 1969.

Serial No. NIDR-82 (c) (68)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Submucous Fibrosis

Previous Serial Number: NIDR-84 (c)

Principal Investigator: Dr. J. E. Hamner, III

Other Investigators: None

Cooperating Units: Dr. Fali S. Mehta and Dr. Dinesh Daftary, Tata Institute of Fundamental Research, Bombay, India

Man Years:

Total:	1 1/4
Professional:	1/4
Others:	1

Project Description:

Objectives:

1. To determine if chili powders can produce submucous fibrosis in the buccal mucosa of hamsters.

2. To study histochemical changes in the lamina propria of patients suffering with submucous fibrosis.

3. To study any ultrastructural changes in collagen of tissue specimens of submucous fibrosis.

Methods Employed:

1. 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.

2. Biopsy specimens from 54 Indians afflicted with submucous fibrosis, were stained with hematoxylin and eosin, van Gieson, Rinehart, Masson trichrome, periodic acid-Schiff, and crystal violet methods

to determine any variance in histochemical staining reaction.

3. Fresh tissue specimens will be fixed with oxmium tetroxide and blocked in Epon for electron microscopic study.

Major Findings:

1. The collagen tissue immediate subjacent to the basal cell layer of the overlying epithelium in the 54 submucous fibrosis biopsies revealed a paler, unusual staining reaction with the van Gieson and Rinehart stains for collagen.

2. After 12 months the hamsters are beginning to show initial histologic signs of submucous fibrosis.

3. The materials have not yet arrived for ultrastructural study of the altered collagen which appears pale, homogenous, and non-bundular with conventional microscopic study.

Significance to Dental Research:

Submucous fibrosis is an oral disease peculiar to Indians. 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 pre-malignant 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.

The altered staining reaction with the collagen stains is a helpful sign in the microscopic diagnosis of early submucous fibrosis.

Proposed Course of Project:

1. The hamster chili studies will continue for another year or the animals' demise.

2. Electrophoresis studies on the collagen will be done on fresh tissue sent from India. Electron microscopy will also be done on the fresh tissue.

Part B Not included

Serial No. NIDR-83 (c) (69)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Implantation of Teeth

Previous Serial Number: None

Principal Investigator: Dr. J. E. Hamner, III

Other Investigators: None

Cooperating Units: Southwest Foundation for Research and Education, San Antonio, Texas

Man Years:

Total:	1/8
Professional:	1/8
Others:	0

Project Description:

Objectives:

1. To observe clinically, radiographically, and histologically in time sequence the response of the surrounding periodontium to autogenous reimplantation of teeth in baboons and to compare those findings with the findings involving autogenous reimplantation of human teeth.

2. To study the feasibility of implanting plastic teeth in the primate as a substitute for lost natural teeth.

Methods Employed:

1. Reimplantation Project: Under general anesthesia lateral incisor teeth would be extracted from 1 of the 10 adult baboons and receive immediate standard endodontic treatment. Being kept moist in normal saline, the tooth would have its root tip removed, and the cementum on the distal side of the root would be disked to remove it. Then it would be reinserted in its socket in less than 20 minutes and stabilized by interproximal plastic bridging. At intervals of 2 weeks, 1 month, 4 months, 6 months, and 12 months the tooth would be X-rayed, then removed in block section which would extend from the lateral incisors' distal alveolar bone to the mesial of the

central incisor. These jaw block specimens would be decalcified and stained with hematoxylin and eosin, Rinehart, Masson trichrome, and the Brown and Brenn methods for microscopic examination.

2. Plastic Teeth Project: Similar procedures were followed as described in (1) above except a quick-cured plastic replica of the natural tooth was made and inserted in the socket with stabilization.

I am the Project Officer for Contract P.H.-43-67-1476 with the Southwest Foundation. The project, "Reimplantation of Baboon Teeth," runs from 6/27/67 to 6/26/69. The estimated support is $15,000 for fiscal year 1969, and the contract will be renewed.

Major Findings:

1. Reimplantation Project: The reimplanted teeth exhibited eventual resorption of the distal surface, commencing after 6 months and becoming more marked after 12 months. The mesial side, however, maintained a good periodontal union between alveolar bone and tooth cementum 12 months after reimplantation with only microscopic foci of resorption. Actual root resorption on the distal surface was caused by clastic cells derived from mesenchymal cells in the periodontal membrane in response to cell-fixed antibody produced by the massive infiltration of plasma cells and lymphocytes.

2. Plastic Teeth Project: This project has not yet extended far enough to obtain sufficient data.

Significance to Dental Research:

The study of the actual causes of the eventual resorption of natural teeth is of vast clinical importance to dentistry because this is the problem a practicing dentist must deal with when faced by anxious parents and a sobbing child holding an avulsed tooth. This study of natural tooth reimplantation revealed the actual cause, namely odontoclasia of the root induced by the cell-fixed antibody produced by plasma cells. It is advocated that a human reimplanted tooth be kept moist while extraorally, an immediate root canal must be done, the root cementum and periodontal fibers should not be scraped off, and the tooth should be reimplanted and stabilized out of occlusion in less than 30 minutes, if possible, for the best results.

A successful replacement for lost or badly broken down teeth has been the goal of many researchers. Plastics appear to offer the most hope at the present.

Proposed Course of Project:

1. Reimplantation Project: A similar experimental model will be carried out to reimplant natural teeth, then study them over specified time intervals, except the animals will be kept on steroid therapy (Celestone) to suppress the inflammatory reaction.

2. Plastic Teeth Project: In collaboration with Falcon Plastics in Los Angeles, California, exact plastic tooth replicas will be made of the extracted baboon teeth and will have a negative electrical charge placed on the root prior to implantation to see if periodontal union can be achieved.

Part B

Publications:

1. Hamner, J. E., III, Reed, O. M., and Stanley, H. R.: Reimplantation of teeth in the baboon. J.A.D.A. (Accepted for publication).

Serial No. NIDR-84 (c) (68)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Leukoedema

Previous Serial Number: None

Principal Investigator: Dr. J. E. Hamner, III

Other Investigators: None

Cooperating Units: Tata Institute of Fundamental Research, Bombay, India; Royal Dental College, Copenhagen, Denmark.

Man Years:

Total:	1/8
Professional:	1/8
Others:	0

Project Description:

Objectives:

1. To study cases of leukoedema in Bolivia; its relationship to the habit of chewing coca leaf, and the possible relationship of coca chewing to inducement of premalignancy in the buccal mucosa of its users.

2. To study cases of leukoedema found in the Indian states of Gujarat, Kerala, Andhra Pradesh, and Bihar; to compare the findings in Bolivia and India.

Methods Employed:

1. Under local anesthesia 46 buccal mucosal biopsies were performed on 39 Aymara and 7 Quechua Indian tin mine workers in the highlands of Bolivia. Thirty-six males and ten females ranging in age from 17 to 75 years were biopsied. The tissue specimens were studied microscopically after being stained with hematoxylin and eosin, Masson trichrome, van Gieson, crystal violet, and the periodic acid-Schiff methods. Twenty normal buccal mucosal specimens were stained by similar methods and used as a base line control.

2. Twenty-six buccal mucosal biopsies were taken on rural Indian villagers who exhibited leukoedema. These tissue specimens will be stained with a similar battery of stains as listed above and studied microscopically.

Major Findings:

1. Leukoedema was a prominent finding on the buccal mucosal side in which the coca leaf/lime chew was held in the Bolivian Indians. Large spongy/vacuolated epithelial cells were noted in the stratum spinosum in a majority of the samples. No clinical or histologic evidence was found to support the idea that leukoedema may be a premalignant lesion. None of the patients displayed buccal carcinoma in spite of lengthy exposure to coca leaf and calcium hydroxide.

2. The histologic material from India has not yet been processed.

Significance to Dental Research:

Studying leukoedema from two vastly different regions of the world should contribute to our basic knowledge of this clinical entity. Evidence points to the conclusion that leukoedema is probably merely a variant of "normal" buccal mucosa, not a pathologic problem.

Proposed Course of the Project:

It is intended to complete the histologic examination of the Indian cases of leukoedema and compare those findings with the Bolivian ones. Attention will be directed towards learning more about the large spongy epithelial cells.

Part B

Publications:

1. Hamner, J. E., III, and Villegas, O. L.: The effect of coca leaf chewing on the buccal mucosa of Aymara and Quechua Indians in Bolivia. O. Sur., O. Med., and O. Path. (In press), 1969.

Serial No. NIDR-85 (c) (66)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Dental Pulp Studies in Humans and Laboratory Rats

Previous Serial Number: NIDR-85 (c)

Principal Investigator: Dr. S. Kakehashi

Other Investigators: Dr. H.R. Stanley and Dr. R.J. Fitzgerald

Cooperating Units: Indiana University Foundation
Indianapolis, Indiana

Man Years:

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

Project Description:

Objectives:

To determine the range of human and animal pulpal response to various operative procedures.

Methods Employed:

Surgical exposures of dental pulps in germfree and conventional rats were treated with various combinations of obtundents. The healing response at various postoperative time intervals was studied after appropriate histopathologic preparation.

Human pulp study material is presently being prepared by Dr. H. Gilmore, Indiana University Foundation, under collaborative research contract PH 43-66-525.

Major Findings:

1. The use of a combination of steroid and obtundent drugs appears to have no effect on the healing of exposed germfree rat pulps.

2. In human tooth cavity preparations, the steroid obtundent drug formulae appear to minimize the typical pulpitis response to cavity preparation.

Significance to Dental Research:

A continuing study program of pulp biology and its response to various new modalities of operative treatment are necessary to keep pace with the needs of the dental profession.

Proposed Course of Project:

Research contract PH 43-66-525 has been extended through FY 1969 without an increase in funds so that Dr. H. Gilmore of the Indiana University Foundation will be able to fulfill the terms of the contract which require the clinical preparation of human teeth by various techniques of instrumentation and medication.

Part B

Publications:

1. Kakehashi, S., Stanley, H.R., and Fitzgerald, R.J.: The exposed germfree pulp--The effects of topical corticosteroid medication and restoration. O. Surg., O. Med., and O. Path. 27: 60-67, 1969.

Serial No. NIDR-86 (c) (66)
1. Oral Medicine and Surgery
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: A Long Term Study of Periodontal Disease in a Stable, Adult, Male Population

Previous Serial Number: NIDR-86 (c)

Principal Investigators: Dr. S. Kakehashi and Dr. N. W. Littleton

Other Investigators: None

Cooperating Units: District of Columbia Fire Department

Man Years:

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

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 occurrence of destructive periodontal disease is being assessed by direct observation of volunteers of the D. C. Fire Department. Examinations are to be repeated 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 Prematurities, (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 have been temporarily delayed. 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) (68)
1. Oral Medicine and Surgery
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: A Study of Differences in Occurrence of Dental Caries in Caucasian and Negro Children

Previous Serial Number: None

Principal Investigators: Dr. S. Kakehashi and Dr. N. W. Littleton

Other Investigators: Dr. R. J. Fitzgerald

Cooperating Units: Dr. Finn Brudevold, Forsyth Dental Clinic, Boston, Massachusetts

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description:

Objectives:

1. To determine dental caries experience and clinical evidence of fluorosis in samples of Negro and Caucasian children.

2. To determine if differences in dental caries experience and fluorosis in these groups were associated with corresponding differences in:

   a. Recovery of caries-inducing streptococci from samples of dental plaque.

   b. Acidogenicity of samples of dental plaque.

   c. Enamel fluoride levels.

   d. Oral hygiene status.

   e. Selected dietary characteristics.

Methods Employed:

Participants in this study were 106 Caucasian and 109 Negro males, 13 to 14 years of age, from similar socio-economic backgrounds, who were continuous residents of an area supplied with water augmented to 1 ppm fluoride. Each participant was examined for dental caries, oral hygiene, fluorosis and periodontal status. Samples of plaque were collected for the recovery and enumeration of hamster-type caries-inducing streptococci as well as acidogenic potential of the plaque. An enamel biopsy of a single tooth was taken for calcium and fluoride determinations. Information regarding each participant's dietary characteristics as to frequency of eating and sugar intake were evaluated.

Major Findings:

1. Caucasian boys had twice as many decayed, missing and filled teeth as Negro boys (DMF means were 3.5 and 1.7 respectively).

2. Specific caries-inducing streptococci were recovered from 30 percent of the cultures of plaque obtained from Caucasians as compared to 17 percent for Negroes.

3. No differences were recorded in the mean pH of suspensions of plaque material in both groups.

4. Negro boys reported eating more frequently and eating sweet foods more often than the Caucasian boys.

Significance to Dental Research:

This study was undertaken in an attempt to explain the differences in dental caries experience between Negroes and Caucasian residents of the same geographic area, who were exposed to the same 1 ppm fluoride water supply.

Proposed Course of Project:

1. Studies pertaining to the collection of enamel biopsies are still in progress.

2. To further delineate caries and fluorosis differences in Negroes and Caucasians, a study of fluoride excretion patterns following the ingestion of known amounts of fluoride in Negroes and Caucasions with and without evidence of fluorosis will be undertaken.

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, 1968 through June 30, 1969

Part A

Project Title: Post-surgical Tissue Healing

Previous Serial Number: NIDR-87 (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:

To correlate age of patient, type of impacted tooth, systemic condition of patient and surgical trauma with postoperative 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. This study has continued but the results have not been reviewed. Approximately 100 additional impacted third molars have been done under this study but some have not been included because of lack of controls within the same patient. It is doubtful whether significant information will be gained because of patient variation and response.

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, 1968 through June 30, 1969

Part A

Project Title: Sectional Roentgenographic Study of the Temporomandibular Joint Following Bilateral Osteotomy of the Ramus of the Mandible

Previous Serial Number: NIDR-88 (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. Post-operative 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 post-operative. 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:

Three additional cases have been completed. Old cases are being followed as they are available for recall. No new major findings are recorded at this time.

Part B not included.

Serial No. NIDR-90 (c) (67)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Evaluation of Premedication in Conjunction with Local Anesthesia in Oral Surgical Procedures

Previous Serial Number: NIDR-89 (c)

Principal Investigator: Dr. P.M. Lightbody

Cooperating Units: None

Man Years:

Total:	0
Professional:	0
Other:	0

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 Finding:

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. This study has not yet been initiated. It will be revised, both in technic and drugs, and hopefully started in the coming year.

Part B not included.

Serial No. NIDR-91 (c) (44)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

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, Anticariogenic Effect of Fish Protein Concentrate

Previous Serial Number: NIDR-94 (c)

Principal Investigator: 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. Some pH measurements are also made in these areas and salivary flow rates are measured.

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.

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. Usually F.P.C. contains from 100 to 250 ppm. fluoride. This fluoride accounts for part of the anticariogenic effect. (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.) Experiments are underway comparing the anticariogenic properties of usual high fluoride F.P.C. with a newly prepared low fluoride F.P.C., i.e., F.P.C. containing less than 100 ppm. fluoride.

Some of the anticariogenic effect is 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, and a comparison of the relative effect of these factors with the fluoride factor is being made.

Significance to Dental Research:

The chief significance of the clinical studies is to point up the problems 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 anti-cariogenic 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 92 (c) (66)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Studies of Oral and Pharyngeal Form and Function in Infants

Previous Serial Number: NIDR 95 (c)

Principal Investigator: Dr. J. F. Bosma

Other Investigators: Dr. M. Mainen

Cooperating Units: National Institute of Child Health and Human Development (Dr. W. Bell and Dr. J. Weiffenbach); Division of Radiology, Clinical Center, NIH (Dr. R. Pierce); Medical Arts and Photography Branch; Biomedical Engineering and Instrumentation Branch, NIH; Department of Anatomy, University of Maryland School of Medicine.

Man Years:

Total:	1¼
Professional:	¼
Other:	1

Project Description:

Objectives:

To describe development of form and functions of pharynx and mouth in the human infant.

Methods Employed:

Anatomical studies of dissected or sectioned cadaver fetuses and of disarticulated or sectioned skulls of fetuses, by standard and laminographic radiography drawings. Microsections of tongue, of soft palate, of eustachian apparatus and of larynx excised from near-term cadaver fetuses which are photographed, diagrammed and drawn.

The sensorimotor function of the infant mouth which is under study is that of orientation and tongue following elicited by touch stimuli. This is observed by cinema as infant is held; his eyes are covered.

Major Findings:

Anatomy. The skeleton and musculature of the basicranium, face and pharynx of the fetus near term are being portrayed and described. Ten of twenty five fetal skulls selected from an assortment in a biological supply firm are under study by photography and radiography. Five of these will have radiolaminography in coronal plane by Dr. R. Pierce, Clinical Center Diagnostic Radiology Department, and then be imbedded and sawn in close approximation to the plane and level of laminography. The cut sections will be photographed, drawn and labelled in parallel with the radiolaminographs. Five of the skulls will be papainized and disarticulated, and the individual bone measured, photographed, drawn and labelled. These description methods of sawn sections and disarticulated bones are comparable to those employed in the book, Development of the Rat Head Skeleton (see Report #93).

Function:

Our current concerns are with the orienting and approximating functions of the front of mouth. In initial experiments, we have cinema demonstrations of lateralward motions of tongue and head, following touch stimulation to side of tongue tip. These elicitations by "contiguous" stimulation are analogous to tongue responses following "discontiguous" stimulation, as in the rooting reflex (studies and observations by Mysak).

Significance to Dental Research:

Definition of norms of anatomy of the skeleton and musculature will facilitate definition of somatic abnormalities in the human neonate. This distinction of abnormality will be particularly meaningful as the criteria are radiologic. For the physical examination of the neonate's facial and pharyngeal area is peculiarly liable to errors, because of the fat panniculus which sheaths the baby generally (and its face particularly), and the comparative inaccessibility of the infant's pharynx and dorsal part of mouth to inspection and to palpation. Anomalies of skeleton and musculature of the newborn are commonly undetected or mis-estimated.

The sensorimotor functions of the infant's mouth and pharynx should be evaluated by a battery of multiple, separate criteria.

Their actions in feeding, in cry and in orientation and following, apparently vary in sensory guidance, as well as in central representation and in pattern of immediate motion. It is possible that the adaptations of somatic skeletal and muscular form are particularly related to the individual infant's sensory-cued orienting and positional mechanisms, rather than to his functions of feeding.

Proposed Course of Project:

Completion of current skeleton anatomical studies and publication of an atlas style volume: The Head Skeleton of the Human Neonate. Completion of 21 current general illustrations and 37 detail illustrations of the musculature of face, mouth and pharynx. And inclusion or combination of these with the illustrations of skeleton in a more general volume: The Mouth and Pharynx of the Human Infant: Skeleton and Musculature.

Amplification of these studies of function by addition of Dr. Weiffenbach to this Section.

Part B not included

Serial No. NIDR 93 (c) (66)
1. Oral Medicine and Surgery
2. Oral & Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Studies of Development of the Head Skeleton of the Rat

Previous Serial Number: NIDR-96 (c)

Principal Investigator: Dr. J. F. Bosma

Other Investigators: None

Collaborating Units: University of Michigan School of Dentistry (Dr. M. Baer and Miss E. Hirshfeld); University of Pennsylvania School of Dentistry (Dr. J. Ackerman); National Library of Medicine - by a Special Publication Grant; Colorfax Laboratories, Inc.

Man Years:

Total:	1¼
Professional:	¼
Other:	1

Project Description:

Objectives:

Demonstration of patterns of growth of the head skeleton of the rat, as a representative mammal.

Methods Employed:

Alizarin-staining of skeleton of growing rats, sacrifice and saw-sectioning of head skeleton, and photography and artist illustration of the sawn sections, are completed. Photographs of 152 stained bone sections have been duplicated in 1500 sets of 120 2 x 2 projection transparencies by the Colorfax Laboratories, employing novel adaptations of color reproduction processes, for accurate duplication of alizarin staining and other bony details.

Other rat head skeletons of selected ages, not alizarinated, are papainized and disarticulated, and the individual bones photographed in standard fashion in relation to metric scale.

Textual commentary on the sections and bones, and the Chapter on History of Developmental Osteology, Methods in Developmental Osteology, and Principles of Developmental Osteology are in preparation.

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 Summer of 1969. The distinctive projection transparencies of this volume have been completed.

Successive alizarinization and sacrifice of part of a series of pigs and of sheep is completed and the skulls are now ready for sectioning. The study will also be extended to rabbits.

Part B

Publications:

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 content of this Symposium has appeared as a single issue (July, 1968) of the American Journal of Physical Anthropology.

Serial No. NIDR 94 (c) (62)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Studies of Sensory and Motor Functions in Subjects Impaired by Malformations or Neurological Disease

Previous Serial Number: NIDR-97 (c)

Principal Investigator: Dr. J. F. Bosma

Other Investigators: None

Cooperating Units: National Institute of General Medical Sciences (Dr. D. Brodie); National Heart Institute (Dr. R. Henkin); National Institute of Child Health and Human Development (Dr. J. Kavanagh); University of Indiana Medical Center (Dr. B. Weinberg); University of Kansas Medical Center (Mrs. Ann Wornall); NIH Medical Arts and Photography Branch; The Johns Hopkins Medical Center (Cleft Palate Clinic).

Man Years:

Total:	¼
Professional:	¼
Other:	0

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.

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 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.

Part B

Publications:

1. Bosma, J. F. and Brodie, D.: Disabilities of the Pharynx in Amyotrophic Lateral Sclerosis, as Demonstrated by Cineradiography, Radiology, 92:97-103, 1969.

2. Bosma, J. F. and Brodie, D.: Cineradiographic Demonstration of Pharyngeal Area Myotonia in Myotonic Dystrophy Patients, Radiology, 92:104-110, 1969.

3. Henkin, R. I., Christiansen, R. L. and Bosma, J. F.: Facial Hypoplasia, Growth Retardation, Impairment of Oral Sensation and Perception and Hyposmia: A New Syndrome, in Second Symposium on Oral Sensation and Perception, in press. Thomas:Publ.

4. Weinberg, B. and Bosma, J. F.: Severe Hypoplasia of the Tongue, J. of Sp. & Hear. Dis., 1969.

5. Weinberg, B. and Bosma, J. F.: Glossopharyngeal Respiration and Esophageal Speech, J. of Sp. & Hear. Dis., in press.

Serial No. NIDR 95 (c)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Studies of Oral Sensation and Perception

Previous Serial Number: None

Principal Investigator: Dr. J. F. Bosma

Other Investigators: None

Cooperating Units: National Institute of Child Health and Human Development (Dr. J. Weiffenbach); Pennsylvania State University, Department of Speech and Hearing (Dr. E. McDonald); University of Kansas Medical Center, Division of Speech and Hearing Disorders (Dr. R. Shelton and Mrs. Ann Wornall); University of Indiana Medical Center (Dr. B. Weinberg).

Man Years:

Total:	¼
Professional:	¼
Other:	0

Project Description:

Objectives:

To understand the mechanisms of the separate modalities of oral sensation, the interaction of these modalities, their guidance of oral area motor function, and the mechanisms of the acquisition of subjective experience from the mouth.

Methods Employed:

We continue to develop methods and criteria for evaluation of oral sensation and perception. Our current concern is with touch-elicited motions of the tongue, and associated orienting motions of the head. A proximity probe has been developed which signals touch by illumination of its point and also by a tone signal on the cinema record of the testing procedure or process. Cinema demonstrations have been prepared, and those methods are under evaluation at two universities.

We continued to find and study patients having abnormalities of sensa- and perception. These are for the most part individual examples. As of a child with hypoplasia of ventral portion of tongue (and tongue contours of "tongue tie") who also has coincident disability of oral speech articulation and failure of approximation of tongue tip to either contiguous or discontiguous stimulation. Or a man with spastic dyscoordination who has entirely competent speech but almost complete disability of other voluntary functions of the mouth.

A particular function and role of our research effort in this field has been the assembling and assortment of pertinent information from related fields of neurophysiology, psychology, neurology and speech specialty. This has been accomplished by a series of conferences and symposia, the publication of a first Symposium on Oral Sensation and Perception and the arrangement and editorial preparation of a second Symposium.

Significance to Dental Research:

The several separate functions of the mouth are best differentiated and defined by their elicitations and the subjective experiences which they occasion. The separate postnatal developments of each of these functions are associated with changes in their associated sensory guidance and subjective experience. Thus, oral sensation and perception affords the best clues and criteria of postnatal development.

Proposed Course of Project:

Study methods and criteria of oral elicitations and responses of the newborn infant are accumulating rapidly. Studies of oral feeding function in puppies and other mammalian young particularly in the Section on Developmental Psychology, NIMH, provide a homologic reference for work on human newborns.

The general topic of oral sensation and perception in school age children and adults is under intensive study, and some standard methods and norms have been evolved. The battery of tests and criteria is being extended and expanded as a result of work in the fields of psychology, speech specialty and neurology.

The major problem area currently remaining is that of studies of sensory elicitation and guidance and of general response of the infant and the young child. The early genesis of our distinctively human functions of feeding, of positional maintenance, of speech and other derivative manipulations occurs in this period of development. The reason that we understand these mature categories of function so poorly, and are so inadept in definition and therapy of their disorders, is that we have so little understanding of the sensory guidance of these functions in their nascent stages, and of the mechanisms by which these nascent performances become stabilized or established in their central representations.

The next Symposium on Oral Sensation and Perception, scheduled for November 23-25, 1970, will include reports and discussions of studies in young infants, as well as in school age children. We hope to have our first consecutive studies in infants ready by that time.

Part B

Publications:

1. Bosma, J. F.: Editor's Comment, Second Symposium on Oral Sensation and Perception. In press, Thomas:Publ.

2. 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 press, Thomas:Publ.

3. 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 press, Thomas:Publ.

4. Henkin, R., Christensen, R. H., 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 Press, Thomas:Publ.

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, 1968 through June 30, 1969

Part A

Project Title: Study of Taste Thresholds, Tastebud Distribution, and Associated Dentofacial Form

Previous Serial Number: NIDR 100 (c)

Principal Investigator: Dr. R. L. Christiansen

Other Investigator: Dr. J. F. Bosma

Cooperating Units: Dr. R. I. Henkin, N.H.I.

Man Years:

Total:	½
Professional:	½
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 Lidocain 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 not included

Serial No. NIDR 97 (c) (65)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Study of Oral Area Motor Mechanisms by Use of Displacement and Pressure Transducers

Previous Serial Number: NIDR-101 (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; Mr. Karlind Moller, School of Dentistry, University of Minnesota

Man Years:

Total:	½
Professional:	½
Other:	0

Project Description:

Objectives:

1. To further improve performance and reliability of intra-oral displacement and 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 studies 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 onset 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.

Significance 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.

Proposed Course of Project:

Continued instrumentation development and intensive analysis of intra-oral pressure patterns during speech and swallowing activity; and study normal and abnormal movements of the soft palate during speech and other functional tasks.

Part B not included

Serial No. NIDR 98 (c) (68)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Restitution of Mandibular Form After Condylar Injury

Previous Serial Number: NIDR-102 (c)

Principal Investigator: Dr. P. J. Coccaro

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1½
Professional:	½
Other:	1

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. To 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 three 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 seven-year-period demonstrate a dramatic diminution of facial asymmetry present when patient first came to clinic.

2. Initial laminographs of temporomandibular 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 mandibule before treatment. Final laminographs of temporomandibular 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. Others have had many surgical procedures to correct the size disparity on one side with the other.

Part B

Publications:

1. Coccaro, P. J.: Restitution of mandibular form after condylar injury in infancy (a 7-year study of a child), Am. J. Orthod., 55: 1, 32-49, January, 1969.

Serial No. NIDR 99 (c) (68)
1. Oral Medicine and Surgery
2. Oral and Pharyngeal Development
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Clinical and Roentgenographic Analysis of Orthodontics (on a Continuing Basis) in Cleft Palate Habilitation

Previous Serial Number: NIDR-103 (c)

Principal Investigator: Dr. P. J. Coccaro

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	½
Professional:	½
Other:	0

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 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.

Part B

Publications:

1. Coccaro, P. J.: Orthodontics in Cleft Palate Children Habilitation (A Continuing Process). Accepted for publication in the Cleft Palate Journal.

Serial No. NIDR-100 (c) (68)
1. Oral Medicine and Surgery
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Prenatal Development of the Larynx; Human and Comparative Investigation

Previous Serial Number: NIDR-104 (c)

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:	1/2
Professional:	1/2
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 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.

Methods Employed:

1. Review of histologic material of normal laryngeal development in the human, rat, and chick.

2. Nine-day (post implantation) rat neural crest tagging using tritiated thymidine for autoradiographic mapping.

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. Malcolm 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 rat larynx.

2. Anticipate publication of a review article on the development of the human larynx entitled: Human Larynx: Neural crest contribution to the larynx in the rat.

Part B not included

Serial No. NIDR-101 (c) (65)
1. Oral Medicine and Surgery
2. San Francisco, California

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Immunological Studies in Recurrent Aphthous Stomatitis

Previous Serial Number: NIDR-81 (c)

Principal Investigator: Dr. E.A. Graykowski

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	2
Professional:	1
Other:	1

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 hyposensitization 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 precipitation reaction, total serum proteins and albumin-globulin ratio, serum electrophoresis, immunoglobulins, and urinalysis.

2. Lymphocyte transformation tests are done on aphthous patients and controls by culturing lymphocytes from both groups and determining the amount of transformation to blast stage stimulated by antigens from the causative organism (aphthous streptococcus 2A), various

related and unrelated antigens, and a non-specific stimulant. The degree of stimulation is measured by counting in a scintillation counter the amount of tritiated thymidine incorporated into the cells during the transformation to blast cells.

3. The laboratory experimental model for aphthous stomatitis is the albino, female guinea pig (Hartley strain), which is sensitized to the causative agent (Streptococcus sanguis). Hyposensitization of these animals is attempted by means of the intravenous and subcutaneous injection of a streptococcal vaccine prepared from heat-killed Streptococcus indifferent, an organism antigenically related to the causative agent.

4. A colorimetric method is used to determine the amount of cryofibrinogen present in the heparinized plasma of aphthous patients, and an attempt is being made to relate the presence of an abnormal amount of this substance to the production of the oral and skin lesions observed in these patients.

5. 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. The transformation of the lymphocytes from aphthous patients in cell culture is suppressed by the antigen (from aphthous streptococcus 2A) when compared to the control (nonaphthous) patients' lymphocytes.

2. The lymphocytes from aphthous patients respond in a normal manner to other streptococcal antigens and nonspecific stimulants.

3. 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 proportional to the severity of the disease in the patient tested.

4. The injection of an antigenically related streptococcal antigen (Streptococcus indifferent) intravenously over a period of eight weeks reduces the delayed skin reaction in guinea pigs sensitized to the aphthous streptococcus 2A and hyposensitization is further verified by decreased pyrogen reactions to the aphthous Streptococcus 2A CHO antigen injected intraperitoneally in the hyposensitized animals indicating a beneficial effect from repeated injections of a streptococcal antigen.

5. A direct correlation has been made between the presence of an increased amount of cryofibrinogen in the plasma of aphthous patients and the presence of an acute exacerbation of the disease. The amount of cryofibrinogen decreases rapidly upon the administration of cortico-steroids and lesser amounts are present during remissions of the disease.

Significance to Dental Research:

The finding of suppression of lymphocyte transformation in aphthous patients by a specific antigen from the causative organism may account for the lack of an antibody response to the presence of the aphthous streptococcus 2A in the oral tissues. This decreased lymphocyte transformation in aphthous patients may be useful as a diagnostic test and also provide a means of measuring a patient's response to treatment with a vaccine.

Proposed Course of Project:

A major effort will be made to develop an accurate diagnostic test for aphthous patients, and in addition the investigations will be directed toward demonstrating the presence of specific antibodies against the aphthous streptococcus 2A in the circulating lymphocytes of aphthous patients. Several methods of hyposensitization will be investigated in order to determine the most feasible method of controlling this disease.

Part B

Publications:

1. Bosma, J.F., Graykowski, E.A., and Trygstad, C.W.: Chronic Ulcerative Pharyngitis, Archives of Otolaryngology 87: 103, 1968.

2. Barile, M.F., Francis, T.C., and Graykowski, E.A.: Streptococcus sanguis in the Pathogenesis of Recurrent Aphthous Stomatitis. In Guze, L.B. (Ed.): Microbial Protoplasts, Spheroplasts and L-Forms, Baltimore, Md., Williams & Wilkins Co., 1968, pp. 444-456.

3. Graykowski, E.A.: Management of Aphthous Stomatitis. In Current Dermatological Management, Maddin, S. (Ed.), C.V. Mosby Co., St. Louis, Mo., (in press).

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 125 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. Studies are now in progress to obtain submaxillary saliva by cannulation of Wharton's duct in normal subjects and patients with cystic fibrosis.

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 an 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 21 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. Preliminary results indicate no statistically significant greater incidence of high arch palates in congenital heart disease patients compared to normal control patients.

A prime example of a laboratory experiment extended to the human research environment is that of dextranase in the control of the adherent dental plaque. Reports of laboratory tests on hamsters by NIDR scientists indicate the significant reduction in smooth surface plaque formation when the hamster is exposed to dextranase.

Six patients with dextran-producing streptococci were selected for a pilot study to determine the effect of dextranase on the reduction of the adherant plaque on the teeth. These patients rinsed with a 25% sucrose solution at regular intervals for three days, followed by staining and photography to document the accumulated plaque. After dextranase rinse, the teeth were restained and photographed.

The results of this preliminary study indicate a decrease in plaque on teeth following the use of the dextranase mouth wash. Tests are continuing with the potential of developing methods to prevent or minimize the formation of dental plaque, thus reducing the risk of smooth surface caries.

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 1967	Fiscal Year 1968	Fiscal Year 1969 (Estimated)
Admissions to Clinical Center	4,076	4,047	3,960
Visits	14,759	13,047	11,952
Examinations	1,510	1,506	1,536
Treatments	31,259	32,860	29,484

The reduction in admissions to the Clinical Center for fiscal year 1969 is reflected in the decrease of visits, examinations and treatments in the Dental Services Branch. The shortage of personnel resulting from the limitation on replacing position vacancies also contributed to the reduction in the number of treatments rendered.

Serial No. NIDR-102 (c) (65)
1. Dental Services Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Reaction of the Human Dental Pulp to Cavity Preparations and Filling Materials

Previous Serial Number: NIDR-105 (c)

Principal Investigator: Dr. H. Swerdlow

Other Investigators: Dr. H. 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. (See Part B - Publications)

b. 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.

c. 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. (See Part B - Publications)

d. 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 eugenol. 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 72 teeth in 19 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 391 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.

6. The physical properties of gallium-palladium-tin alloy exhibit superior strength, resistance to flow, thermal expansion coefficients, wetting tooth structure resulting in tighter marginal adaptation compared to dental amalgam. The biological evaluation performed at the NIH showed no evidence of corrosion. The pulp reactions were similiar to those normally obtained with conventional dental amalgam which usually show a small percentage (less than 5%) of severe lesions.

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), (Adaptic, Blendant)

3. Testing a pit and fissure sealant to inhibit caries when using such formulas as isobutyl 2-cyanoacrylate monomer in a quartz base.

4. Silicate - an anterior restorative material.

5. The value of a varnish cavity liner under amalgam restorations to reduce the established trauma from amalgam insertion.

6. The value of new temporary filling materials improving, sedation, sealing and insulation.

Part B

Publications

1. Stanley, H. R., Swerdlow, H., Stanwich, L., Suarez, C.: A Comparison of the Biologic Effects of Filling Material With Recommendations For Pulp Protection. Gold Foil Journal (in press)

2. Waterstrat, R. M. Evaluation of a Galium-Palladium-Tin Alloy for Restorative Dentistry, JADA 78:536-541 March 1969

Serial No. NIDR-103 (c) (68)
1. Dental Services Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1968 through June 30, 1969

Part A

Project Title: Palatal Vault Measurements in Patients with Congenital Heart Disease

Previous Serial Number: NIDR-106 (c)

Principal Investigator: Dr. B. Goldman

Other Investigators: None

Cooperating Units: 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 comapred to that of normal control patients and analyzed for statistical correlation.

1-2

Major Findings:

The main phase of this pilot study has been completed and the results are presently being analyzed. Preliminary analysis indicates that there is not a statistically significant greater incidence of high arched palates in congenital heart disease patients compared to normal control patients.

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-one patients were included in this pilot study. The further course of the project will be determined upon analysis of the forthcoming final results.

Part B not included

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