LETTER OF TRANSMITTAL.

Sir: I have the honor to transmit herewith a paper on "The relation of bacteria to the flavors of Cheddar cheese," being a report of the investigations made by Mr. Lore A. Rogers, expert in dairy bacteriology in this Bureau. The general plan and scope of this work was determined by the chief of the Dairy Division, and the experimental portion was conducted under the supervision of the chief of the Biochemic Division. I recommend that this paper be published as a bulletin of this Bureau.

Respectfully,

D. E. Salmon,
Chief of Bureau.

Hon. James Wilson,
Secretary of Agriculture.

Dy.—54.

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ILLUSTRATION.

THE RELATION OF BACTERIA TO THE FLAVORS OF CHEDDAR CHEESE.

By Lore A. Rogers,

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INTRODUCTION.

Although cheese making has been carried on for many centuries, it is only in recent years that satisfactory scientific explanations have been offered for methods in use for ages. The impetus given by the work of Pasteur to biological research in general, and particularly to the study of fermentations, led naturally to investigations into the causes of cheese ripening. The first and most plausible explanation, that this change was brought about by bacteria and other micro-organisms, obtained such wide acceptance that it is still considered the correct one by all except those familiar with the most recent work. As this question came to be more carefully studied it was found that this theory was insufficient, for certain kinds of cheese at least, and that the complex changes taking place in the milk solids between the time the cheese is started in the vat and its sale as a food can be brought about only by a combination of causes. Our knowledge of these causes and the conditions governing them has been greatly advanced in the last few years by the work done in this country at the agricultural experiment stations in Wisconsin and New York (Geneva), and by Freudenreich and Jensen in Switzerland. While this work has gone a long way in clearing up the more obscure points, there is still much to be explained, and any data that will add to our somewhat limited stock of information will hasten the time when cheese making will be conducted entirely on scientific principles rather than by rule of thumb.

In this country we are especially interested at present in the ripening of Cheddar cheese, or, as it is more commonly called, American factory cheese, as this is the only kind made here on an extended scale. This kind of cheese is differentiated by its low water content from the soft cheeses of the Limburger and Brie type. The high water content of the soft cheeses, by making them a favorable medium for the growth of many kinds of organisms, changes the nature and rapidity of the fermentation. The American Cheddar resembles, in its method of manufacture, composition, and fermentations, the English Cheddar and the European Emmenthaler, or Schweizer.

The study of these fermentations is exceedingly complex, involving not only a great variety of biological questions, but also extensive

changes of the most obscure natute in the proteid compounds which make up the great bulk of the cheese curd. The most important of the final products of this digestion is, as we shall see later, a group of compounds known as amides.[a] Some of these have their own peculiar, penetrating odor and taste. Many of them are, as their names indicate, found in decaying organic matter, but certain of the amides which give putrefying material its characteristic odor are not found in cheese. Thus the ripening of cheese, while resembling in many ways, especially in the highly flavored cheese of the Limburger type, the ordinary putrefaction of proteid matter, is distinguished from it by certain of the end products, as well as by the quantities in which the amides and ammonia are formed.

Before going into a discussion of the experimental work it will be well to mention briefly the changes taking place in ripening cheese, and to review our present knowledge of the causes which produce these changes. By the ripening we do not mean simply the development of certain desirable flavors. While these are essential, they are only the final result of a long series of complex changes which must take place before the tough, indigestible curd becomes edible; all of these changes are a part of the ripening and must be understood before we can hope to explain or control the production of flavors. In studying the ripening of cheese we have to do with one group of compounds only. The sugar, while it is rapidly broken up and may under certain circumstances be connected with certain abnormal fermentations, breaks down most rapidly into comparatively simple acids which have little direct influence on the flavor. On the other hand, many of the decomposition products of fat (which is an important constituent of Cheddar cheese) have an especially pungent odor and taste. However, it is well established that the change in the fat is, at most, very slight (Weidmann).[1b] It is in the nitrogenous constituents—the casein, or, more properly, the paracasein—that we find the important changes.

In carrying on the work recorded in this paper the writer has received many valuable suggestions from Mr. Edwin B. Hart, associate chemist at the New York Agricultural Experiment Station. In the manufacture and initial bacteriological examinations of Cheeses IV and V, the bacteriological laboratory and the dairy of the New York Agricultural Experiment Station at Geneva were used. The many courtesies in this connection are acknowledged.

PHYSICAL CHANGES.

The changes in the physical condition of the curd are marked. The cheese as it comes from the press has the spongy consistency of a mass

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[a] In this paper, under the term amide, are included all nitrogenous compounds not precipitated by tannic or phosphotungstic acid, except ammonia.

[b] See bibliography at end of bulletin.

of rubber, but may be broken into rough bits. During the ripening it becomes soft and waxy, until in a well-ripened cheese it may be spread with a knife like good butter. A thin rind is formed on the surface by the drying out of the curd and by the growth of molds and other organisms. This is always very thin and has no influence on the texture or flavor beyond a few millimeters from the surface.

CHEMICAL CHANGES.

The chemical changes in the nitrogenous constituents of the cheese are profound and affect very deeply its general appearance, its digestibility, and its flavor and aroma. In the milk nearly all of the nitrogen is in the form of casein floating in the serum as a fine suspension, which is coagulated by rennet, forming paracasein and thus greatly concentrating the proteids by separating from them a large part of the water. It has always been assumed that in the ripening process this substance was broken down directly into simpler bodies soluble in water. In a recent paper embodying the results of careful and exhaustive research, Van Slyke and Hart[2] show that there is formed in the early stages of the manufacture a monolactic acid salt of the paracasein. During the "ripening" of the milk and the heating of the curd after cutting, the milk sugar is split up into lactic acid by the acid-forming bacteria normally present in the milk or introduced as a starter, and the acid thus set free unites with the paracasein forming the unsaturated or mono-acid salt, if the acidity is normal; or a saturated or di-acid salt, if the acidity is abnormally high. This body is insoluble in water but soluble in dilute solutions of sodium chloride. In normal cheese it is present in quantities varying from 40 to 75 per cent of the total nitrogenous constituents. It has not been conclusively proved that the formation of lower compounds is at the expense of this body, but its gradual decrease and the proportional increase of water-soluble substances, during the ripening, point strongly to this assumption. This point is well illustrated in Table I, taken from Van Slyke and Hart's paper.

TABLE I.—*Amounts of salt-soluble and water-soluble products in a cheese at different ages.*

[From Am. Chem. Jour. XXVIII, 1902, p. 417.]

Age of cheese.	Per cent nitrogen of total nitrogen in cheese.	
	Nitrogen in form of compounds soluble in salt solution.	Nitrogen in form of water-soluble compounds.
1 day	58.7	6.78
1 month	42.4	19.30
3 months	33.4	26.02

As the ripening proceeds there is a gradual transition of the nitrogen from the more complex to simpler compounds. A number of bodies intermediate between the higher and lower proteids are formed, but only in small quantities. Albumoses and peptones are always present in considerable quantities, but in a normal cheese the nitrogen shows no tendency to accumulate in this form; the decomposition keeps pace with the production, resulting in an accumulation of amides, and to a less extent of ammonia. In a thoroughly ripened Cheddar cheese 3 or 4 per cent of the total nitrogen will be in the form of albumoses, and an equal amount in the form of peptones, while the amides will contain about 30 per cent of the nitrogen and the ammonia 3 to 5 per cent. As will be pointed out later, the rapidity of these changes depends directly, conditions of manufacture being equal, on the ripening temperature. While little experimental work bearing directly on this question has been done, the tendency is to believe that the flavor of cheese is due to the presence of the amido compounds, probably influenced to some extent by the ammonia. The gradual development of the flavors peculiar to Cheddar cheese appears to be closely associated with the accumulation of amides and ammonia, and probably has no direct connection with the presence of any of the other compounds in the cheese. On the contrary, the amounts of the more complex nitrogenous compounds are always smaller after the flavors have developed.

It is now well known that the intensity of the flavor may be more or less controlled by regulation of the temperature of the curing chamber. As a general rule the higher temperatures produce a more pronounced flavor. Chemical analyses of cheese cured at different temperatures show that the influence of a higher ripening temperature is most noticeable in the larger proportion of amides and ammonia to total soluble nitrogen. The sharpness that always occurs in cheese ripened at high temperatures is probably due, to some extent, to their high ammonia content[3, p. 216]. On the other hand, cheese ripened at low temperatures has a clean but mild flavor and a correspondingly low amount of ammonia. In a valuable contribution to the study of cheese flavors, Van Slyke and Hart[3] have shown the probability of certain changes among the amido bodies themselves that may account for at least a part of the changes that the flavor undergoes as the cheese ripens. They found in an examination of cheeses at different ages, differences in the kinds of amido bodies present that probably represented a transition from one class of compounds to another. In a cheese four and one-half months old they found lysatine, histidine, and lysine; in one fifteen months old, tetramethylenediamine (putrescine) and lysine. In discussing the results of their work they say:

There appears to be good evidence that there is regularly in the cheese-ripening process, in the case of hard cheeses like Emmenthaler and American Cheddar, a

conversion of primary into secondary amido compounds; and these chemical changes may explain, perhaps, the gradual development of flavor in normal cheese; in other words, we may find that the changing flavor of cheese, as it ages, is due, to some extent, to increasing quantities of secondary amido compounds.

To summarize: The chemical changes in ripening cheese consist essentially in the formation of a lactate of paracasein with a subsequent conversion into simpler compounds soluble in water, with a gradual accumulation of amides and ammonia. Certain of the amides themselves are probably still further broken up into simpler amides and into ammonia.

CAUSES OF THESE CHANGES.

LACTIC-ACID BACTERIA.

What is probably the first step in the breaking down of the soluble curd is brought about indirectly by the activity of bacteria. As has already been mentioned, the formation of compounds soluble in water is preceded by the union of lactic acid with the casein or paracasein, the acid having been produced through the fermentation of lactose by the lactic-acid bacteria. These are normally present in milk in large numbers, but their action is usually hastened by the addition of a "starter," which is practically a nearly pure milk culture of one or more species of the lactic-acid-producing bacteria. During the process of manufacture these bacteria attain to enormous numbers and in a short time cause a marked increase in the acidity. The proper degree of acidity is estimated roughly by cheese makers by touching a small piece of curd to a hot iron and observing the fine threads that appear as the curd is pulled away from the iron, the length of the string increasing with the acidity. Van Slyke and Hart have shown this property to be due to the presence of the lactate of paracasein. The action of the bacteria in this connection is entirely indirect, and cheese could doubtless be made without bacterial fermentation of the sugar by carefully adding small quantities of acid. Van Slyke, Harding, and Hart,[4] in making cheese in which the bacteria were suppressed from the beginning by the addition of chloroform, produced the acid salt of paracasein by the gradual addition of lactic acid.

PEPSIN.

It has been shown by Babcock and Russell[5] that pepsin is present in rennet extract, and is incorporated in the cheese in quantities sufficient to affect the ripening changes very appreciably. This was also proved, independently, by Orla Jensen[6] working with Emmenthaler cheese. The former scientists give the results of extensive investigations, showing that the products resulting from the use of pepsin or an increased amount of rennet are the higher proteids characteristic of pepsin digestion.

TABLE II.—*Showing amounts of different nitrogenous decomposition products in cheese 6 months old, made with varying quantities of rennet.*

[From Seventeenth Annual Report, Wisconsin Agricultural Experiment Station.]

Amount of rennet per 1,000 pounds milk (ounces).	Per cent nitrogen of cheese in form of—					
	Total soluble.	Albumoses.	Peptones precipitated by—		Amides.	Ammonia.
			Tannin.	Phosphotungstic acid.		
3	1.36	0.20	0.21	0.08	0.70	0.17
9	1.60	.42	.25	.08	.69	.16
18	1.88	.68	.27	.07	.70	.16

It will be seen from this table that an increased amount of rennet—that is, an increase of pepsin—resulted in an increase of soluble nitrogen confined to albumoses and higher peptones, while the lower peptones precipitated by phosphotungstic acid, the amides and the ammonia remained practically unchanged. While pepsin doubtless has more or less to do with the breaking down of the paracasein, it is quite evident that it can have only an indirect influence on the flavor, because its activity is confined entirely to the production of the higher products which play little or no part in giving the cheese its peculiar taste and aroma.

GALACTASE.

A distinct advance in the knowledge of cheese ripening was the discovery by Babcock and Russell [7] of a proteolytic enzyme inherent in milk, to which they gave the name galactase. This is secreted with the milk, and, becoming incorporated in the cheese, plays an active part in the general decomposition of the casein. In describing this enzyme Babcock and Russell [7, p. 179] compared it to trypsin, on account of the end products of its digestion and its accelerated action in an alkaline medium, but in a later report they [8] stated that it is differentiated from trypsin and related to the bacterial enzymes by its ability to form ammonia.

It has been pointed out by Freudenreich [9] that the increase of amido nitrogen in galactase digestion is unimportant, and his conclusions have recently been confirmed by the work of Van Slyke, Harding, and Hart [4, p. 244]. The latter compared the progressive decomposition of normal cheese with that of cheese made to simulate as closely as possible the normal Cheddar, but in which the activity of all factors except pepsin and galactase had been excluded by the use of chloroform. It had been previously shown that the proteolytic action of galactase was only slightly if at all depressed by the presence of chloroform, even in large quantities. The results of their work bearing on this question are summarized in Table III.

TABLE III.—*Showing the progressive decomposition of normal cheese and of cheese ripened by pepsin and galactase only.*

[Adapted from Bulletin 203, New York Agricultural Experiment Station.]

Age of cheese (months).	Per cent nitrogen of total nitrogen in form of—					
	Albumoses and peptones.		Amides.		Ammonia.	
	Normal.	Chloroform.	Normal.	Chloroform.	Normal.	Chloroform.
1	2.95	3.71	5.42	0.86	0.86	0.00
1¼	2.51	7.31	8.49	1.82	1.29	.00
3¼	5.37	10.20	12.60	3.22	2.51	.00
5¼	4.97	12.40	18.50	4.73	3.38	.00
7	3.08	10.90	20.10	8.11	4.42	.00
9	2.70	12.52	23.50	11.60	4.87	.00

An examination of this table shows a striking difference in the amounts of amido nitrogen in the normal and in the chloroform cheeses. At the end of three and one-half months the normal cheese contained nearly four times as much amido nitrogen as the chloroform cheese. This proportion was gradually reduced by the slow accumulation of amides in the chloroform cheese, but at nine months, when the normal cheese was thoroughly ripe, there was still twice as much amido nitrogen in the normal as in the chloroform cheese. On the other hand, the chloroform cheese contained, at nine months, nearly five times as much nitrogen in the more complex forms of albumoses and peptones as the normal cheese. Even more noticeable is the entire absence of ammonia in the chloroform cheese, forming a marked contrast to the normal, which at the end of nine months had nearly 5 per cent of its nitrogen in the form of ammonia.

The inability of galactase to form ammonia is even more distinctly shown by the digestion of casein in milk, cited in the bulletin referred to [4, p. 225]. In this case the enzyme was working under very favorable conditions, yet while the amount of amides was comparatively high, ammonia was not formed. From these results it is safe to conclude that, while galactase may and probably does play an important part in the conversion of paracasein, or more probably paracasein lactate, into soluble compounds, these compounds are confined to the higher proteids—the albumoses and peptones—with only a slight formation of amides and none of ammonia.

This forces upon us the necessity of accounting for certain changes in normal cheese that can not be produced by the activity of galactase and pepsin alone. In other words, the presence of those compounds, the amides and ammonia, which probably give cheese its peculiar flavor, is due, partially at least, to the activity of a third factor which has not yet been definitely determined. The accelerating effect of high ripening temperatures on the rate and amount of change, and especially on the amounts and nature of the lower compounds, has already been

noted. The increased production of these simpler bodies may be due (1) to the accelerating effect of more favorable temperature on the enzymes present in the cheese; (2) to an increase in the numbers of bacteria normally present; or (3) to a change in the nature of the bacterial flora. The improbability that this increase in simple digestion products is brought about by the increased activity of galactase is illustrated by the comparison of the products of galactase digestion with ripe cheese given on page 165 of the Sixteenth Annual Report of the Wisconsin Experiment Station. In this case, although the enzyme was working in milk under most favorable temperature conditions (37–38° C.), yet in 112 days it had converted only about 17 per cent of the total nitrogen into the form of amides, while in normal cheese, where the conditions would be much less favorable to enzymic activity, at approximately the same age, over 16 per cent was in this form. The entire absence of ammonia formation, even under most favorable circumstances, as noted above, makes this possibility even more remote.

The fact that the production of these compounds is suppressed by the presence of anesthetics, as has already been shown, and to some extent at least by low temperatures, would indicate that their presence was the result of vital activity. The causal relation between bacteria and the ripening of cheese, both in regard to the solution of the paracasein and the production of flavor, has long been maintained by different investigators. This could be brought about by bacteria, either directly through the metabolism of the living cells or indirectly through the action of enzymes secreted by them. In the latter case the action would be continued after the bacteria had entirely disappeared. Even with bacteria present in large numbers it is difficult to explain by the direct action of cells the extensive proteid decomposition that takes place in cheese. The amount of nitrogenous matter actually needed by bacteria for food is exceedingly small and a decomposition becomes marked only when, through the action of an excreted enzyme, the proteid is broken down in quantities greatly in excess of the needs of the organism. It is likely that the insoluble curd could be extensively changed by bacteria only through the agency of such an enzyme; and the probability of the conversion by intracellular action of the soluble bodies already formed by galactase and pepsin into the lower amides and ammonia in the quantities usually found in cheese seems remote.

In studying the relation between bacteria and the ripening of cheese, it is not sufficient, as some investigators have seemed to think, to isolate bacteria from cheese and test their ability to produce, when grown in pure culture in milk, cheese-like flavors and aromas. As Freudenreich has pointed out, cheese ripening is essentially a decomposition and the flavor is due to certain simple decomposition products not peculiar to cheese but belonging to proteids in general; these

bodies may be formed under favorable conditions by many species of putrefactive bacteria. The conditions existing in milk, and especially in milk containing a pure culture, are entirely unlike those existing in hard cheese, and results obtained in this way can not be logically applied to the whole problem of cheese ripening. To prove that any particular group or species of bacteria is the cause of cheese ripening or of any specific phase of it, it is necessary to show not only that these bacteria are present in the cheese, but that at the time these changes occur they are present in numbers sufficiently large to account for the phenomena, and that they are able, under similar conditions, to produce similar changes; if the bacteria have been present at an earlier stage, but have disappeared before the changes in question occur, it must be demonstrated that they secrete an enzyme capable of producing these results. With the complex conditions existing in cheese it is not easily possible to observe all of these rules with anything approaching exactness, but with our present knowledge of cheese ripening it is possible, by artificial fermentations, to secure results from which we may safely draw conclusions applicable to cheese-ripening problems.

THE DIRECT ACTION OF BACTERIA.

Sufficient work has already been done on the bacteria in various kinds of cheese to throw considerable light on this part of the problem. In Europe, especially, a great deal has been written on the relation of bacteria to cheese ripening. Most of the European bacteriologists have followed Duclaux in ascribing the essential rôle to bacteria of the liquefying class. Duclaux's[10] conclusions were based on the ability of certain bacteria, which he called the "Tyrothrix," to digest milk so as to produce cheese-like flavors. Freudenreich has shown repeatedly that while the lactic bacteria develop rapidly in Emmenthaler cheese and soon attain enormous numbers, the liquefying bacteria of the Tyrothrix, or hay bacillus group, find in hard cheese unfavorable conditions and never multiply even when artificially introduced in large numbers. The lactic-acid bacteria increase rapidly for a short time, and a slow decrease follows. Old Emmenthaler, even when showing signs of putrefaction, contains a comparatively small number of bacteria.[11]

The only exhaustive quantitative bacteriological study on American Cheddar cheese is that of Russell and Weinzirl.[12] They found a slight decrease in the total number of bacteria in the first day or two after making, followed by a rapid increase. The maximum was reached in 4 to 24 days, depending apparently on the temperature at which the cheese was kept, and then began a decline, rapid at first, but soon becoming more gradual and continuing as long as the cheese was examined. The increase was confined to the lactic-acid bacteria

with sometimes a slight increase in the gas formers. The digesting bacteria did not increase, but, on the contrary, soon almost completely diappeared.

Work done by Harrison,[13] partly at the Wisconsin Station and partly in Canada, shows that these results are correct for American Cheddar in general; by studying the bacterial flora of three Canadian cheeses for an extended period, he obtained results confirming, with a few minor exceptions, the conclusions of Russell and Weinzirl. On the basis of these results, together with those of Freudenreich, which are doubtless applicable to all hard cheese, the only bacteria capable of directly influencing cheese ripening are those of the lactic-acid-forming group. This necessarily assumes for this group the ability to effect extensive changes in the cheese proteids. It has been maintained by Freudenreich that the lactic-acid bacteria, or at least certain varieties which he finds predominating in Emmenthaler cheese, possess this ability. By growing them in sterile milk, in which their action was prolonged by neutralizing the acid formed with calcium carbonate, he found a decided increase in the amount of soluble nitrogen, of which a large part was in the amido form.[14] This work has not been confirmed. In repeating these experiments Nicholson,[15] working at the University of Wisconsin with lactic-acid bacteria from cheese, was unable to detect any appreciable increase of soluble nitrogen in milk cultures in which the acid had been neutralized with sodium carbonate.

It has also been suggested that the digestion of milk casein was not comparable to the digestion of paracasein. Chodat and Hofman-Bang[16] tested the digestive ability of lactic-acid bacteria by growing them in a medium made by coagulating milk with rennet, washing until Fehling's solution failed to give a test for sugar, drying and using about $1\frac{1}{2}$ grams with a suitable amount of water. By removing the sugar the inhibitory effect of the acid was avoided and the bacteria were allowed to grow unchecked for 81 days. At the end of this time there was no appreciable increase in soluble nitrogen. This work is, however, open to criticism in that the removal of the sugar prevented the formation of the first decomposition product and possibly the further digestion of the paracasein.

Freudenreich's[17] hypothesis of the relation of lactic-acid bacteria to the ripening of Emmenthaler cheese is based largely on his experiments in which cheese made from heated milk, or from milk in which the bacteria had been largely excluded by aseptic conditions, ripened normally only when lactic-acid bacteria developed. While Van Slyke and Hart do not maintain that the acid salt is an essential step in the digestion of casein by enzymes other than pepsin, they do show that it is digested in normal cheese ripening. It is probable that this is a necessary step in the breaking down of the curd and it is possible that Freudenreich's cheese failed to ripen, not because the direct action of

the lactic bacteria was wanting, but because of the absence of the lactate of paracasein formed indirectly by this group of bacteria.

In the paper mentioned above, Freudenreich admits the probable action of galactase, and probably a liquefying coccus, in the digestion of the curd; but still maintains that the production of the lower by-products, and consequently the flavor, is due to the action of the lactic bacteria. If, as Freudenreich evidently assumes, the lactic bacteria change peptones and other soluble proteids into amides and ammonia without the agency of an enzyme, cheese held at a comparatively high temperature, where the ripening is more active, should show a larger number of lactic bacteria than cheese made under identical conditions but held at a temperature low enough materially to check the ripening. If other bacteria play a direct part in forming amides and ammonia there should be a differentiation, qualitative or quantitative, in the bacterial flora of the two cheeses. Babcock and Russell,[18] in a paper on the effect of differences in temperature on the ripening of Cheddar cheese, discuss the comparative flora of high and low temperature cheeses, without, however, going into details. In both cheeses the lactic-acid bacteria were soon predominant. The development was slower in the cheese cured at low temperatures but persisted for a longer time, while in the high-temperature cheese the numbers were greatly diminished and persisted for a shorter time. They also state that there was a qualitative differentiation and suggest that this has possibly a relation to the change in flavor.[a]

BACTERIAL FLORA OF CHEDDAR CHEESE.

CHEESES I AND II.

The work which has already been done on the bacteria of cheese leaves little question as to the general nature of the flora of hard cheese. It has seemed advisable, however, to go over a part of the ground again in connection with a general investigation of the changes and flavors of cheese. Certain phases of the subject have been selected for review and especially the effect of temperature on the flora and its possible connection with the flavor. As the material for beginning this preliminary investigation, two cheeses were obtained from Cook Brothers, of Lewis County, N. Y., made in their factory under commercial conditions from purchased milk. They represented normal American factory cheese in every way, except being of the Young America size, and consequently liable to become abnormally dry. This was prevented by giving them a heavy coating of paraffin soon after they were received. The two cheeses were made together and

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[a] Since this paper was written, the results of an extensive investigation on this question have been published by Harrison and Connell.[19] These confirm, in a general way, the results given in this paper.

separated only at the press. They were shipped on the third day after making and were 7 days old when received at the laboratory.

Cheese I was placed in a refrigerator at a temperature of 10° to 12° C., where it remained for about one month, when it was changed to a specially constructed curing box, in which the temperature varied from 5° to 10° C. Cheese II remained at room temperature (20° to 25° C.) for 1 month, when it was transferred to a curing box so constructed that it could be held at a constant temperature of 23° C. Cheese II ripened rapidly. When only 36 days old it was already well ripened; at 50 days it still had a mild flavor, but at 80 days the flavor was sharp. It deteriorated very rapidly after reaching its prime until at 123 days it was so strong, both in flavor and odor, as to be unfit to eat. On the other hand Cheese I ripened very slowly. At 81 days it still had a distinctly unripe taste, but when 123 days old it had developed a very fine mild flavor.

The difference in the composition of the two cheeses, when matured, is given in the following table:

TABLE IV.—*Per cent nitrogen of cheese in Cheeses I and II when 123 days old.*[a]

In the form of—	Cheese I.	Cheese II.
Total soluble	0.85	2.00
Albumoses and peptones	.35	.38
Amides	.42	1.38
Ammonia	.08	.24

a In making the chemical analyses given in this paper the methods given by Van Slyke and Hart[20] were followed. The amides and ammonia were determined in the filtrate from tannic-acid sodium chloride precipitate, and the albumose and pepton nitrogen was obtained by difference. The latter thus includes all proteids precipitated by tannic acid.

The essential difference is found in the high amide and ammonia content of the highly flavored cheese, compounds which, as has already been pointed out, are characteristic of bacterial digestions rather than of digestion by pepsin or galactase.

The bacterial flora of these cheeses was studied from the time they were received until they were completely ripe, not, however, with any attempt to separate and describe the various species present. While this would be desirable, it is doubtful if the value of work of this nature, especially when the present condition of systematic bacteriology is considered, is sufficient to repay the great labor involved. The object of this part of the investigation was to determine if differences in temperature resulted in a differentiation, qualitative or quantitative, in the bacterial flora and, if any differentiation occurred, whether or not it bore any relation to the differences in flavor. For this purpose it was enough to determine the total number of bacteria and to separate them in a general way into groups.

In the bacteriological examination of cheese certain peculiar difficulties are encountered that render the most carefully obtained results

more or less inaccurate. Slight variations in the texture or the mechanical arrangement of the curd at the time of pressing cause an unequal distribution of the bacteria, and this difference is intensified by the relatively small sample that can be used. An even more serious difficulty is the extremely large dilutions that must be used, especially when the cheese is still young and the number of lactic-acid bacteria is large. If one uses a dilution large enough to allow a count of the lactic colonies, there will be at most only an occasional colony of the less numerous forms and an accurate count can not be made. On the other hand, if a small dilution be used the lactic colonies will be so numerous as to inhibit the growth of all species less resistant to acid. However, exact numerical results are not essential. It is sufficient if we know what species occur and approximately the numbers present.

The results of the bacteriological examinations of Cheese I and Cheese II are given in Tables V and VI. The ordinary method was employed of grinding a weighed sample of the cheese with sterile sugar and diluting with water. For the lactic-acid bacteria, lactose gelatin was used with the addition of sterile litmus solution, and for the nonlactic forms the ordinary beef extract pepton gelatin. The yeasts were determined by adding to each tube of gelatin sufficient tartaric acid to inhibit completely the growth of bacteria.

TABLE V.—*Bacteria and yeasts per gram in Cheese I, ripened at 5° to 10° C.*

Age (days).	Total bacteria and yeasts.	Lactic-acid bacteria.	Yeasts.	Liquefying bacteria.
7	84,770,000	84,570,000	200,000	....
16	22,199,000	21,970,000	229,000	....
22	4,471,000	4,445,000	18,000	8,000
36	4,210,700	4,164,000	46,000	700
50	2,772,500	2,717,000	55,700	800
60 a	....	....	....	4,200
81	453,800	438,000	11,600	6,100
123	3,913,650	3,904,000	7,050	2,600
382	50,200	45,600	....	4,600

a Alkaline gelatin.

TABLE VI.—*Bacteria and yeasts in Cheese II, ripened at 20° to 25° C.*

Age (days).	Total bacteria and yeasts.	Lactic-acid bacteria.	Yeasts.	Liquefying bacteria.
7	66,676,000	66,360,000	316,000	....
16	3,147,000	3,143,000	4,000	....
22	546,000	538,000	5,550	2,500
36	167,000	164,000	200	2,900
50	289,900	289,000	....	900
60 a	....	....	....	1,600
81	10,800	9,200	....	1,600
123	42,500	42,000	....	500

a Alkaline gelatin.

The results given in Tables V and VI, agree in a general way with those of Russell and Weinzirl, and Harrison. The flora proved to be made up almost entirely of lactic-acid-forming bacteria. Yeasts were present in considerable numbers, but decreased slowly in the low-temperature cheese and more rapidly in the one held at the high temperature.

The total bacteria.—The bacteria had reached their maximum at or before the time the first examination was made. In Cheese I the decrease was rather rapid for a short time and then very gradual up to 123 days. The recorded increase at that time was not actual, but was due to the presence on these plates of a large number of extremely small lactic colonies. The plates on this date were plain pepton gelatin without the addition of litmus, while in the earlier plates, made with considerable litmus, these colonies, on account of their small size and weak acid formation, were probably entirely overlooked. At this time they made up nearly the entire acid-forming flora. There were in both cheeses a comparatively small number of inert forms of various kinds, but as they were present in unimportant numbers no accurate count was made. A large number of subcultures were made from this group from time to time, but there was no particular predominating form and no differentiation in favor of either cheese.

In Cheese II the decrease was very rapid, the total falling in the second week from 66,000,000 to 3,000,000, and in the third week to about 500,000. It is probable that these numbers are somewhat low, as the small lactic colonies mentioned under Cheese I were present in considerable numbers at 123 days. These colonies were almost microscopic, and an accurate count could not be made. However, their number was estimated at 2,000,000 to 3,000,000 per gram. They were designated as the bb type. Subcultures were made from these colonies and the effect of this species on bouillon and on litmus milk was observed. In the bouillon the growth was at the bottom only. Milk was curdled very slowly, and the litmus changed to pink. There was no digestion.

The liquefying bacteria.—In the first two sets of plates made with the dilutions necessary for the large number of lactic bacteria, no liquefying colonies appeared. In the later examinations plain pepton gelatin plates were made with small dilutions, and liquefying bacteria were found in comparatively small numbers. It was found that the number of liquefiers could be more accurately determined by adding to each tube of 6 or 7 cubic centimeters of pepton gelatin 1 cubic centimeter of N/10 NaOH. This amount is sufficient to inhibit greatly most of the acid-forming species without materially retarding the liquefying forms. Counts obtained by such methods are necessarily inaccurate, as some of the liquefiers would probably be inhibited by a strongly alkaline medium. In these two cheeses, at least, the liquefying bac-

teria persisted in small but appreciable numbers for some time, and did not, as both Russell and Harrison have stated to be the case in hard cheese, almost completely disappear in a short time. These were not entirely, as might be expected, colonies of spore-forming bacteria of the hay bacillus type, but included, especially in the first few weeks, many colonies of a coccus forming a small saucer-shaped liquefaction, with a round white or straw-colored colony in the center. The total number of liquefiers decreased slowly in both cheeses and, like the other groups, more rapidly in Cheese II.

<p style="text-align:center">CHEESES IV AND V.</p>

In continuance of the same line of investigation, a similar examination was made of two cheeses made under the personal supervision of the writer at the New York Agricultural Experiment Station on the 29th of January, 1903. The mixed milk of two days was used. Part of this was held overnight without cooling, in order that the bacteria might develop in a normal manner. At this time, on account of the recent loss of the station barns by fire, the herd was stalled in temporary sheds. Notwithstanding these unfavorable surroundings, the milk was in better than average condition, as the bacteriological examination shows. The milk was ripened by Hansen's dry lactic ferment, and the cheese was made by the usual Cheddar method. At the time of pressing the curd was separated into two equal parts and pressed as two Young Americas, each weighing about 9 pounds. On the following day they were taken from the press and shipped by express. They arrived in Washington twenty-four hours later, and were transferred at once to the curing boxes, Cheese IV, at 23° C., and Cheese V in the one held at 5–10° C. Gelatin plates were made from the milk before adding the starter and from each cheese after taking from the press and at short intervals up to 133 days.

For some unknown reason these cheeses ripened very slowly. In Cheese IV, when 77 days old, while the curd was apparently well broken down, there was very little flavor, and 20 days later, although some advancement was shown, it was still only slightly developed. Cheese V at 77 days still had the flavor of green cheese, although the texture showed considerable change. Twenty days later there was apparently a slight increase in flavor. The progress of the ripening is well illustrated by the chemical analysis made at 77 days and given in Table VII.

TABLE VII.—*Per cent nitrogen in Cheese IV and Cheese V when 77 days old.*

In the form of—	Cheese IV.	Cheese V.
Water-soluble	1.45	0.72
Albumoses and peptones	.99	.51
Amides	.38	.15
Ammonia	.08	.06

The relatively high amount of water-soluble nitrogen and the low amount of amides and ammonia agree with the condition of the texture and flavor at this time. The bacteria were determined as before by the use of lactose-litmus gelatin for the lactic group, and alkaline-pepton gelatin for the liquefiers. Only approximate results can be obtained for the latter group by this method on account of the frequent rapid growth of colonies of the hay bacillus type necessitating a count before the smaller colonies have developed.

TABLE VIII.—*Number of bacteria per gram in milk and in Cheese IV.*

Product and age (days).	Total bacteria.	Lactic group.		Liquefying group.			
		Total.	214 bb type.	Total.	Hay b type.	214 q type.	214 z type.
MILK (10 c. c.) a	435,000	99,000	....	315,000	6,000	309,000	....
CHEESE:							
1	17,103,800	17,090,000	....	13,800	1,800	12,000	....
3	62,015,600	62,000,000	....	15,600	4,800	8,400	2,400
7	29,148,000	29,118,000	....	30,000	6,000	2,000	22,000
11	12,556,100	12,540,000	....	16,100	3,600	....	12,500
15	36,375,700	36,360,000	....	15,700	6,600	....	7,800
35	....	49,800,000	5,600,000	Plates all liquefied.			
42	16,335,200	16,320,000	....	12,800	....	....	....
53	19,418,400	19,400,000	6,040,000	18,400	3,200	....	15,200
65	12,888,200	12,880,000	3,949,000	8,200	....	....	....
77	3,379,000	3,357,000	2,826,000	22,000	6,100	....	15,900
95	1,130,000	1,122,000	794,000	18,000	10,400	....	7,600
116	929,200	914,000	778,000	15,200	7,300	....	7,900
133	366,700	356,000	275,000	10,700	4,900	....	5,800

a Plates made immediately before adding rennet.

TABLE IX.—*Number of bacteria per gram in milk and in Cheese V.*

Product and age (days).	Total bacteria.	Lactic group.		Liquefying group.			
		Total.	214 bb type.	Total.	Hay b type.	214 q type.	214 z type.
MILK (10 c. c.)	435,000	99,000	....	315,000	6,000	309,000	....
CHEESE:							
1	25,905,600	25,860,000	....	45,600	2,400	43,200	....
3	56,625,500	56,600,000	....	25,500	4,100	18,600	2,800
7	36,098,000	36,060,000	....	38,000	8,000	....	30,000
11	25,456,400	25,420,000	....	36,400	10,000	2,400	24,000
15	51,454,000	51,440,000	....	14,000	5,500	4,500	4,000
35	30,417,400	30,400,000	....	17,400	3,000	600	5,400
42	54,793,500	54,774,000	....	10,500	....	....	....
53	55,884,200	55,880,000	3,380,000	4,200	....	....	....
65	50,504,600	50,500,000	1,020,000	4,600	....	....	....
77	56,555,000	56,538,000	14,194,000	17,000	3,800	....	13,200
95	19,607,900	19,600,000	10,180,000	7,900	....	....	....
116	19,104,800	19,100,000	4,950,000	4,800	2,600	....	2,200
133	25,989,900	25,980,000	2,355,000	9,900	6,600	....	3,300

DISCUSSION OF TABLES VIII AND IX.

In these cheeses the separation into groups was carried somewhat further than in Cheese I and Cheese II. Included in the lactic group is the bb type already mentioned as observed in the late stages of the ripening of Cheese I and Cheese II. In the liquefying group are included, in addition to the common hay bacillus, the type 214 q (a coccus forming small white or straw-colored colonies surrounded by a saucer-shaped liquefaction) and the 214 z type, a bacillus forming a very small cup-like liquefaction with a white film of growth over the surface; 214 q is probably identical with the coccus occurring in Cheese I and Cheese II. Each of the two latter types probably included more than one variety.

The total bacteria.—The striking feature of the flora was the long duration of the comparatively high numbers. The maximum number was reached in each cheese at or soon after the third day. This was followed in Cheese IV by a comparatively small drop to a number that was held for a long time. A second decided drop occurred at 77 days in Cheese IV, and at 95 days there was a distinct decrease in Cheese V. This change corresponds in a general way with the change in the flavor. In other words, the development of the flavor-producing compounds and the bacterial changes apparently remained at a standstill for several weeks.

The lactic group.—Since the total is made up almost entirely of lactic bacteria, what has been said of the total will apply also to the latter group. The numbers given for this group, as well as those of the totals, show more or less variation due largely to the erratic growth of the bb type, previously mentioned. Slight variations in the conditions sometimes entirely suppressed the formation of colonies of this type, which in connection with the difficulty of making an accurate count of colonies almost microscopic in size, will account for most of the variation in numbers. There was apparently no real, but rather only a relative, increase of the bb type in the high-temperature cheese. There was in these cheeses a decrease in this group, but it was much slower than that of the other varieties, and at 95 days it made up almost the entire lactic group.

The liquefying group.—Numbers given under this heading can be considered only as rough approximations, indicating in a general way the kinds of liquefiers present and their relative numbers. There was evident a distinct decrease in the total, confined largely to the 214 q type. This coccus decreased with some rapidity in the high-temperature cheese, practically disappearing at 7 days, while in the low-temperature cheese it persisted for about 30 days longer. There was an apparent increase in type 214 z, but this may have been because these colonies developed slowly and were overlooked on the first few sets

of plates.   With those two exceptions this group maintained fairly constant numbers throughout the entire period.

CHEESES VII AND VIII.

A third pair of duplicate cheeses were made for this investigation under commercial conditions by Cook Brothers, of Lewis County, N. Y., and shipped in an ice chest on the second day after making. They were made the last of June and were 5 days old when received, but, as the chemical and bacteriological analyses show, were practically fresh.   Cheese VII was stored in a large refrigerator.   This held ordinarily at about 12° C., but during the hot weather it went a few times above 15° C.   In the latter part of the ripening the temperature ranged between 8° and 12° C.   Cheese VIII was held at a constant temperature of 23° C.   Cheese VII ripened slowly and normally, showing at 90 days a fine, smooth texture and clean flavor, with only a slight sharpness.   At 164 days, although overripe and somewhat sharp, it was still in fair condition.   Cheese VIII, on the other hand, was well ripened when 37 days old.   It had then a clean flavor with only a slight sharpness.   At 93 days it had a strong odor and taste and was entirely unfit for use.   Chemical analyses made from time to time to show the progress of the ripening are given in the following table:

TABLE X.—*Relative rate of ripening of Cheeses VII and VIII.*

Cheese number.	Ripening temperature.	Age.	Per cent nitrogen of cheese in the form of—			
			Total soluble.	Albumoses and peptons.	Amides.	Ammonia.
	° C.	Days.				
VII	10–12	5	0.24	0.14	0.10	0.00
		37	.79	.42	.30	.07
		93	1.07	.61	.40	.06
		136	1.27	.68	.49	.10
VIII	23	5	.21	.15	.06	.00
		37	1.05	.45	.49	.11
		93	1.30	.57	.65	.08
		136	1.50	.56	.75	.19

Bacteriological analyses were made as before with lactose litmus gelatin for the determination of the lactic bacteria and with alkaline gelatin for liquefiers.   The results are given in the following table:

TABLE XI.—*Number of bacteria in Cheeses VII and VIII.*

Age (days).	Total bacteria.	Lactic.		Liquefiers.	
		Total.	Small colonies.	Total.	214 q.
Cheese VII:					
5...............................	157,950,000	156,640,000	....	1,310,000	1,310,000
9...............................	....	114,160,000	....	....	....
16.....	74,682,300	74,680,000	....	2,700	2,300
23...............................	41,620,600	41,620,000	3,600,000	600	600
30...............................	76,901,200	76,900,000	5,120,000	1,200	800
37...............................	40,140,600	40,140,600	....	600	600
88...............................	12,972,200	12,970,000	4,820,000	2,200	2,000
112...............................	1,031,400	1,030,000	....	1,400	1,400
134...............................	637,800	637,000	....	800	400
Cheese VIII:					
5...............................	151,140,000	147,580,000	....	3,560,000	3,560,000
9...............................	90,950,000	90,150,000	....	800,000	800,000
16...............................	84,880,600	84,880,000	....	600	600
23...............................	24,792,000	24,790,000	6,560,000	2,000	1,200
30.....'........................	11,840,000	11,840,000	1,860,000	....	....
37...............................	5,621,200	5,620,000	1,520,000	1,200	800
88........	961,400	961,000	310,000	400	....
112...............................	188,000	188,000	....	....	....
134...............................	12,000	12,000	....	....	....

DISCUSSION OF TABLE XI.

The total bacteria.—In the low-temperature cheese the bacteria decreased slowly, nearly one-fourth of the initial number remaining at 37 days. In Cheese VIII the decrease was comparatively rapid, showing at 37 days only about one-thirtieth of the initial number. At 112 days there were present in Cheese VIII less than 200,000 per gram, while in Cheese VII, at the same age, there were still over 1,000,000. The total was made up, as usual, almost entirely by lactic-acid bacteria. The variety forming very small colonies was present and showed, as before, more ability to resist unfavorable conditions. Otherwise there was no differentiation apparent as the ripening proceeded.

The liquefying bacteria.—The plates made on the day these cheeses were received showed a considerable number of liquefying bacteria of the 214 q type already noted as occurring in each of the four cheeses previously examined. These high numbers dropped immediately, and, although liquefiers were present in both cheeses for some time, they were in small numbers only. Excepting this type, these cheeses were almost entirely free from ordinary liquefiers. The small numbers included under the total were occasional colonies, mostly of the hay-bacillus type.

In addition to these two types there was present, and included with the lactic bacteria, a species liquefying gelatin so slowly that it would ordinarily be entirely overlooked. On the alkaline gelatin plates the

colonies appeared as good-sized lactic colonies. When growing on or near the surface they became surrounded by a very slight depression noticeable only when seen from a sharp angle. On lactose-litmus gelatin plates they appeared only as large lactic colonies surrounded by a red zone. The liquefaction of gelatin seems to be partially inhibited by the presence of lactose. In stab cultures the gelatin is liquefied very slowly, the liquefaction beginning at the surface and extending downward along the line of inoculation. In the course of time the upper part of the gelatin becomes entirely liquefied. On account of the difficulty in distinguishing the colonies of this species from those of the ordinary lactic forms, no separate count was made, but in Cheese VII, when 112 days old, probably about 10 per cent of the total bacteria were of this type. A careful examination of Cheese I, made when it was about one year old, showed that this species was still present in small numbers.

THE EFFECT OF TEMPERATURE ON THE FLORA OF CHEESE.

The effect of the temperature on the flora, as indicated by the six cheeses examined, may be summarized as follows: An increase in the ripening temperature resulted in a rapid decrease in the number of bacteria of all kinds; in the cheeses cured at a low temperature the decrease was much slower, corresponding in a general way to the retarded ripening of the cheese. In other words, the decrease of the bacteria was directly proportional to the rate of ripening. Although a temperature of 23° C. is known to be much more favorable to the development of bacteria than a temperature of 10–12° C., there was in no case, and at no stage of the ripening, an increase in the number of bacteria in the cheeses at the higher temperature. We find also that the variation in the temperature conditions produced no differentiation in the kinds of bacteria present. At no time in the ripening of these cheeses could there be detected in one cheese a species which was not present in its duplicate. In the high-temperature cheeses, where the development of certain kinds of bacteria might be expected, the rapid decrease in numbers was not confined to the lactic bacteria, but included all other species. The only kinds not showing a rapid decrease in numbers were the spore-forming liquefiers. With the possible exceptions of Cheese IV and Cheese V, these were present in small numbers only, and while they did not decrease distinctly there was at least no increase.

These results do not confirm the commonly accepted theory that the undesirable flavor of high-temperature cheese is due to the development of abnormal bacteria. On the contrary, it is evident that, in these cases at least, the undesirable flavor was brought about through some other agency and in all probability by the accelerated action of enzymes which were present in the cheese when it was made or developed in the

earlier stages of the ripening.   The more favorable temperature con-
ditions caused a more active metabolism, which was responsible for, or
at least was coincident with, the rapid decrease in the bacteria. -It is
also evident, both from the chemical analyses and from the develop-
ment of flavor, that important changes were taking place after the
bacteria had reached very low numbers.   This is well illustrated by
Cheese VIII.   In the period between the ages of 93 and 136 days there
was a decided increase in total soluble nitrogen, confined almost entirely,
in so far as the analyses show, to ammonia.   At this time there was
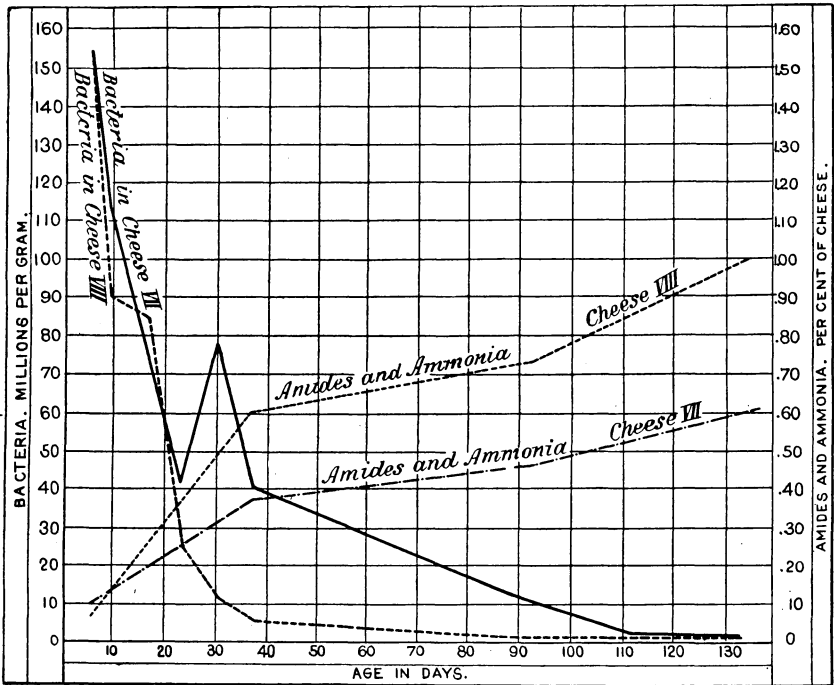present only an insignificant number of lactic-acid bacteria.

Fig. 1.—Diagram showing lack of relation between bacteria and increase of amides and ammonia.

It seems safe to conclude from these results that the bacteria
have little or no direct action in the formation of flavor-producing
compounds.   In this regard this work confirms the views brought
forward in a recent paper by Van Slyke and Hart [21], in which they
compare the carbon dioxide production of a normal cheese with one in
which vital activity had been suppressed by the addition of chloroform.
In the latter carbon dioxide was produced for a short time and then
almost completely ceased, while in the normal cheese it was continued
in considerable quantity for a long period of time.   In the normal
cheese the carbon dioxide production continued steadily for over 200
days and was apparently little influenced by the usual decrease in the

number of bacteria, although, as all work along this line has proved, the bacteria must have reached, in the latter part of this period, very low numbers. In the chloroformed cheese the amido bodies, while present in amounts nearly as great as those of the normal cheese, were confined largely to the primary amides and ammonia was not formed. On the other hand, in the normal cheese the amido bodies were still further broken down into the secondary compounds and into ammonia. They attribute the greater part of the carbon dioxide formation, after the early acid fermentation, to its liberation by the breaking up of the amido bodies, and conclude that this must be brought about by the action, direct or indirect, of bacteria, which are suppressed by the presence of chloroform. There could have been in the milk from which the chlorofrom cheese was made no enzyme able to form the simple decomposition products which these investigators have found to be characteristic of well-ripened cheese.

In view of the results of investigation reviewed and reported herein, it seems very improbable that the production of secondary amides and ammonia in normal cheese is due to the direct action of bacteria. A much more rational explanation is that there is secreted by bacteria, either in the milk before the cheese is made or in the cheese itself during the earlier stages of the ripening, proteolytic enzymes able to break up proteids into secondary amides and ammonia. The next step desirable, therefore, is to consider bacterial enzymes and their source when occurring in cheese.

BACTERIAL ENZYMES.

The secretion of soluble proteolytic enzymes is comparatively common among bacteria, and the causal relation of these enzymes to the ripening of cheese has often been suggested. Duclaux [10], who was one of the first to support the bacterial enzyme theory, based his opinion on the ability of his "Tyrothrix" bacteria to secrete an enzyme, the so-called casease, capable of digesting milk with the formation of cheese-like flavors and aromas. But this theory is supported by so little experimental evidence that it can be considered only as an hypothesis. Freudenreich, as has already been mentioned, has shown that these bacteria occur in normal Emmenthaler cheese in small numbers only and never increase, even when introduced in large numbers. Freudenreich [17], while maintaining the importance of lactic-acid bacteria in the ripening of hard cheese, has suggested the possibility that the enzyme of a certain liquefying coccus plays a part in the decomposition. Jensen [6], in a paper on enzymes in cheese, expresses the belief that certain lactic-acid bacteria isolated from Emmenthaler by Freudenreich are able to secrete a casein-digesting enzyme, but his efforts to demonstrate its existence gave only negative

results.   In the same paper Jensen attempts to demonstrate that bacterial enzymes are developed in Emmenthaler as it ripens.   While his conclusions are probably correct, his methods are open to criticism in that he has assumed that the presence of 0.1 per cent of formaldehyde excludes very largely the action of galactase without materially affecting the action of bacterial enzymes.   Van Slyke, Harding, and Hart [4, p. 230,] have shown that the action of galactase is only partly inhibited by this amount of formaldehyde.   Jensen's work indicates that there exist in ripe Emmenthaler enzymes which are able to increase the less complex decomposition products when the cheese is allowed to digest in the presence of formaldehyde.   These enzymes seem to be absent in young cheese.

In studying the relation of bacterial enzymes to Cheddar cheese ripening, in the investigation here recorded, the writer has employed a somewhat similar method.   At various stages of the ripening samples of 25 grams each were drawn from the cheese, triturated with quartz sand, 196 c. c. water, and 4 c. c. (2 per cent by volume) chloroform added, and the mixture held in sealed flasks in the high-temperature (23° C.) curing box.   Chemical analysis made of duplicate samples gave the initial composition before the digestion.   By eliminating in this way the action of the bacteria and allowing the enzymes to continue their digestion it was possible to obtain some indication of the nature and relative amounts of the enzymes present at successive stages of the ripening.   In results obtained by this method the greatest consideration must be given to increases in the amides and especially in the ammonia.   While galactase may increase the amido nitrogen to some extent, this type of decomposition products is more characteristic of the bacterial enzymes.   The presence of ammonia may be considered a very good evidence that bacterial enzymes are active in the digestion.   Concordant results were obtained from all the cheeses of which bacteriological examinations were made, but, because they were more complete, only those from Cheese VII and Cheese VIII are here recorded.

In Table XII are given the results of an autodigestion of water suspensions made from Cheese VII and Cheese VIII as soon as they were received.   They were at this time 5 days old but were practically fresh cheeses as they had been held most of this time at a low temperature.   The bacteriological examinations showed a large number of lactic-acid bacteria and liquefying cocci.

TABLE XII.—*Digestion in water suspensions, made from Cheese VII and Cheese VIII, when 5 days old.*

Cheese number.	Ripening temperature.	Age of extract.	Per cent nitrogen of cheese in the form of—			
			Total soluble.	Albumoses and peptons.	Amides.	Ammonia.
	° C.	Days.				
VII	10–12	0	0.24	0.14	0.10	0.00
		85	1.60	.57	.96	.07
		134	1.81	.47	1.23	.11
VIII	23	0	.21	.15	.06	.00
		85	1.53	.51	.91	.11
		134	1.62	.51	.99	.12

The digestions from the two cheeses show some differences but these are no greater than would be expected to occur in duplicate flasks from the same cheese, and they may be considered as having been practically indentical at this time. The distinct increase in both the amido and the ammonia nitrogen indicates that bacterial enzymes had already been formed in small quantities. It should be remembered, however, that these enzymes were working under favorable temperature and moisture conditions for a long period of time. Under these conditions we would expect, from a typical bacterial digestion, a more extended decomposition which would be indicated by a higher ammonia content. In cheese ripened at this temperature we would expect to find a considerably higher percentage of ammonia at 134 days, although the small amount of moisture would be much less favorable to enzyme action. This point is well illustrated by the analysis of Cheese VIII made when it was approximately the same age as the water extract on its last analysis. For convenience of comparison these analyses are arranged in another table.

TABLE XIII.—*Comparison of digestion of Cheese VIII and of initial water extract of Cheese VIII.*

	Held at	Age.	Per cent nitrogen of cheese in the form of—			
			Total soluble.	Albumoses and peptons.	Amides.	Ammonia.
	° C.	Days.				
Cheese VIII.............................	....	5	0.21	0.15	0.06	0.00
Extract of Cheese VIII	....	0	.21	.15	.06	.00
Cheese VIII.............................	23	136	1.50	.56	.75	.19
Extract of Cheese VIII.................	23	134	1.62	.51	.99	.12

There was, as might be expected, a somewhat higher percentage of total soluble nitrogen in the water extract. On the other hand there was in the cheese, where the conditions were less favorable, a much higher percentage of ammonia, indicating a deeper decomposition.

The objection may be made that the difference in favor of the cheese may be due to the inhibitory effect of the chloroform contained in the cheese extract.   While little work has been done on bacterial enzymes, it is well known that chloroform has, at most, only a slight inhibitory effect on most enzymes.   In the following table, which gives the results obtained from a digestion made as above from Cheese VIII when it was completely ripe, there is nothing to indicate that the bacterial enzymes were inhibited:

TABLE XIV.—*Digestion in water extract made of Cheese VIII, when 37 days old.*

Age of extract (days).	Per cent nitrogen of cheese in the form of—			
	Total soluble.	Albumoses and peptons.	Amides.	Ammonia.
0	1.05	0.45	0.49	0.11
64	1.76	.37	1.20	.19

In these flasks there was a large increase in soluble nitrogen, with a marked accumulation of amides and ammonia.   In other words, this was a typical bacterial digestion, and when it is compared with the initial digestion given in Table XII must be taken as indicating the elaboration of bacterial enzymes during the ripening of the cheese. If we compare this digestion of Cheese VIII with one made, at the same time, of its duplicate, Cheese VII, held at a lower temperature, we find a weaker power of self-digestion.

TABLE XV.—*Digestion in water extract made of Cheese VII, when 37 days old.*

Age of extract (days).	Per cent nitrogen of cheese in the form of—			
	Total soluble.	Albumoses and peptons.	Amides.	Ammonia.
0	0.79	0.42	0.30	0.07
64	1.52	.47	.95	.10

In comparing these two tables it must be remembered that the initial amounts of by-products were higher in Cheese VIII.   When this is taken into consideration it will be noted that, while the increase in total soluble nitrogen did not differ greatly, the production of amides and ammonia was distinctly greater in the extract from the high-temperature cheese.

This difference is even more marked in the results of digestions made when the cheeses were 93 days old and given in the following table:

TABLE XVI.—*Autodigestion of Cheese VII and Cheese VIII, when 93 days old.*

| | Ripening temperature. | Age of extract. | Per cent nitrogen of cheese in the form of— | | | |
			Total soluble.	Albumoses and peptons.	Amides.	Ammonia.
	°C.	Days.				
Cheese VII.............................	10–12	0	1.07	0.61	0.40	0.06
		97	1.38	.32	.95	.11
Cheese VIII.............................	23	0	1.30	.57	.65	.08
		97	2.12	.30	1.59	.23

It is very evident from these results that the high-temperature cheese possessed much greater power of self-digestion than Cheese VII ripened at a lower temperature. This difference could be explained only by the development of proteolytic enzymes in Cheese VIII during its ripening. From this it seems reasonable to draw the conclusion that, while in the high-temperature cheese, bacterial enzymes were elaborated, forming amides and ammonia, the low temperature at which Cheese VII was held, by inhibiting the vital activity of the bacteria, checked the formation of similar enzymes. It is at least evident that there were, even in the low-temperature cheese, enzymes, in all probability of bacterial origin, able to break down the proteids into the flavor-forming amides and ammonia.

No sharp line can be drawn between the parts of this work done by galactase, by pepsin, and by the bacterial enzymes, but it is certain that the work of the pepsin ended with the peptons; galactase probably produced a considerable part of the higher decomposition products included under the heading "Albumoses and peptons," and part of the amides; the ammonia was, in all probability, the result of the activity of bacterial enzymes. The formation of ammonia presupposes the ability to form amides. This removes the necessity for accounting for the production of flavor through any intracellular action and explains the accumulation of amides and ammonia in cheese with the resulting flavors, after the bacteria have almost entirely disappeared.

SOURCE OF BACTERIAL ENZYMES IN CHEESE.

In considering the source of ammonia-forming enzymes, only two groups of bacteria, the lactic and the liquefying, need be taken into account. This does not mean that the number of kinds of bacteria possibly connected with the formation of flavors in cheese is narrowed down to two species or even to two groups of closely related species. Each of the groups mentioned includes many species, many of them differing widely in most respects but agreeing more or less closely in certain physiological functions.

The lactic-acid bacteria.—Freudenreich and Jensen have long maintained that this group, or at least certain species of this group, digest proteids presumably through the agency of proteolytic enzymes; but they have failed to prove that such enzymes are secreted. The work of Chodat and Hoffman-Bang, and Nicholsen, giving only negative results, has already been cited.

In the bacteriological examinations made of cheese, as already described herein, one species of this group was found which liquefied gelatin very slowly. This action was so slow that it would ordinarily be entirely overlooked and the organism classed with the lactic-acid bacteria. When grown in ordinary milk, an acid curd was formed which remained unchanged. To determine the effect of this organism on milk in which the inhibiting action of the acid had been removed, inoculations were made into flasks of sterile milk containing a small amount of calcium carbonate. The series included also inoculated milk without calcium carbonate and uninoculated sterile milk. All of the inoculated milks curdled. On shaking, the curd separated from the whey, becoming finely divided in the calcium carbonate flasks, while in the others it remained in large tough lumps. Gelatin plates made when the flasks were opened for chemical analysis showed the presence of the inoculating organism in large numbers. At this time all of the inoculated flasks gave a strong reaction with Fehling's solution. The chemical analysis made of these milks when 24 days old is given in the following table:

TABLE XVII.—*Digestion of milk by lactic-acid bacterium 214 dd.*

Treatment.	Per cent nitrogen of cheese in the form of—				
	Total.	Casein.	Total soluble.	Amides.	Ammonia.
Not inoculated, no CaCO₃	0.52	0.46	0.06	0.02	0.00
Inoculated, no CaCO₃		.45	.07	.06	.00
Inoculated, CaCO₃		.33	.19	.11	.00

This organism evidently had a weak proteolytic action when grown in milk containing sufficient calcium carbonate to combine the acid formed, but, on the other hand, had no effect on the casein when the acid was not neutralized. In cheese the duration of the lactic bacteria in comparatively high numbers until the sugar is entirely fermented indicates that a large part of the acid is neutralized. Some may be combined with certain of the ash constituents, but it is probable that the greater part is taken up by the casein. In butter, on the contrary, where the proportion of casein is very much smaller, the lactic bacteria disappear rapidly, leaving a large part of the sugar intact.

The action of the enzyme of this organism on cheese was determined in the following manner: A flask of milk with $CaCO_3$, in which 214 dd

had been growing at 30° C. for 30 days, was filtered through a Chamberland filter. Two lots each of 25 grams of well-ripened cheese were triturated with sand, 171 c. c. water added, and both heated in a steam bath for 30 minutes at 95 to 100° C. Twenty-five cubic centimeters of the filtrate was added to one before heating and an equal amount to the other after heating. Four cubic centimeters (2 per cent by volume) of chloroform were added and the flasks sealed and held at 23° C. The analysis, made at the end of 42 days, is given in the following table:

TABLE XVIII.—*Showing the action of enzyme of 214 dd on sterilized cheese.*

| | Per cent nitrogen of cheese in the form of— | | | |
	Total soluble.	Albumoses and peptons.	Amides.	Ammonia.
Heated enzyme	1.30	0.64	0.53	0.13
Unheated enzyme	1.29	.66	.50	.13

Although a considerable quantity of the digested milk was added to this cheese extract, the amount of enzyme was so small that it caused no appreciable increase of the soluble nitrogen or the decomposition products. In view of the weak proteolytic action of this organism, even under the most favorable artificial conditions, and the exceedingly small amount of enzyme which was secreted under these circumstances, it seems improbable that it could have any important part in the formation of amides and ammonia in cheese. This appears even more improbable when we consider the amount of enzyme of this type which was evidently present in the high-temperature cheese at a comparatively early date.

The liquefying bacteria.—Under this heading is included a large group of bacteria characterized by their ability to peptonize gelatin. It is this group that is most commonly associated with the cheese-ripening processes. In this work two organisms were studied as types of the liquefying bacteria. One of these, 214 x, which liquefied gelatin rapidly, occurred in comparatively small numbers in all the cheeses examined, and may be taken as a type of the resistant bacteria whose spore formation enables them to exist in cheese after the conditions have become unfavorable to growth. The other, 214 q, liquefied gelatin more slowly, and, while it was present in the cheese in large numbers for a few days, it decreased rapidly and in course of time usually entirely disappeared. When these bacteria are inoculated into milk and held at a favorable temperature, their proteolytic action is soon made evident by the coagulation of the milk and the rapid digestion of the curd. There is, apparently, a difference in the nature of the digestion brought about by these two bacteria. In milk inoculated with 214 x the casein is completely digested, the fluid becomes rather dark-colored, and in course of time small bundles of crystals

separate from it. With 214 q the casein is not completely digested
and the clear fluid remains much lighter colored than is the case with
214 x.

In testing the effect of the enzymes of these bacteria on cheese, a
water extract was made, as before, from lots of 25 grams each of
cheese. The enzymes present were destroyed by heating the cheese
one-half hour in a steam bath. To this was added 10 c. c. of the filtrate
obtained by forcing a well-digested milk culture through a Chamber-
land filter. Control flasks were made by heating the filtrate in a
steam bath for 20 minutes before adding it to the cheese extract.
Two per cent by volume of chloroform was added and the flasks were
held at 23° C. The chemical analyses of these cheese extracts, made
after an interval of 90 days, is given in the following table:

TABLE XIX.—*Digestion of cheese extract by enzymes from liquefying bacteria.*

Filtrate from culture of—	Treatment of filtrate.	Per cent nitrogen of cheese in the form of—			
		Total soluble.	Albumoses and peptons.	Amides.	Ammonia.
214 q	Heated	0.96	0.33	0.57	0.06
	Unheated	1.48	.31	1.06	.11
214 x	Heated	.80	.24	.48	.08
	Unheated	2.24	.88	1.22	.14

The filtrate from the milk cultures of each of these bacteria brought
about an extensive change in the composition of the cheese extract.
This is especially noticeable in the increased content of the simpler
decomposition products. In the case of the enzyme from 214 q, the
increase in soluble nitrogen was entirely confined to the amides and
ammonia; the amount of soluble proteids remained unchanged.

It seems highly probable that, in the early stages of the ripening,
various liquefying bacteria secrete, or possibly liberate, by the disin-
tegration of their cells, proteolytic enzymes, which by long-continued
action have a distinct influence on the nature of the by-products and
consequently on the flavor of the ripened cheese. It is unlikely that
this ability is confined to one or even to a very few species, although
it is probable that different species may, by differences in the amount
or nature of the enzyme secreted, produce variations in the product.
These questions can be settled only by a more accurate knowledge of
the kind and proportion of the amides normally present in ripe cheese,
and of the physiology of the bacteria found in cheese.

SUMMARY.

A review of the literature on the ripening of Cheddar cheese shows
that the important changes in the ripening process probably take place
as follows:

First, a combination of lactic acid, liberated by the fermentation of
the milk sugar by the lactic-acid bacteria, with paracasein, forming

the monolactate of paracasein. This compound, insoluble in water, is formed during the manufacture and in the very early stages of ripening.

Second, the gradual transformation of this insoluble body into compounds soluble in water, chiefly albumoses, peptons, and a small amount of the higher amides. This change is probably brought about by the combined action of the two enzymes, galactase secreted with the milk, and pepsin introduced in the manufacture as a contamination of the rennet. The change to this point results in the "breaking down" of the curd and·the softening of the texture, but does not produce the typical cheese flavor.

Third, a gradual accumulation, mostly in the later stages of the ripening, of the numerous compounds of the amide group and their further decomposition into simpler amides and into ammonia. The increase of this group is coincident with, and is usually considered to explain, the increasing intensity of the flavor and aroma. The flavor and aroma are probably still further changed and intensified by a rearrangement of the proportions of the individual amide bodies present.

The presence of this group of compounds in the amounts normally present in cheese can not be explained by the activity of pepsin and galactase. The action of pepsin ends with the peptones; galactase forms amides only in small amounts, even under the most favorable conditions and never produces ammonia. The elimination of pepsin and galactase as possible factors leaves as the most probable cause the action of bacteria, either direct through the metabolism of the cells or indirect through the agency of bacterial enzymes. The bacterial hypothesis is still further supported by the fact that the increase of amides is largely prevented, and that of ammonia entirely, by the presence of antiseptics.

In this paper are given the results of bacteriological investigations made on three pairs of duplicate cheeses; one cheese of each pair was held at a low temperature (8° to 12° C.) and one at a comparatively high temperature (23° C.). In all cases the high-temperature cheeses ripened rapidly and soon developed a strong overripe flavor, while the low-temperature cheeses ripened slowly and retained an agreeable flavor for a long time. The bacteria in the high-temperature cheeses decreased rapidly, and before the cheeses were thoroughly ripened had reached unimportant numbers. The increase in amides and ammonia continued after the bacteria had nearly disappeared. In the low-temperature cheeses the decrease of the bacteria was slower and more gradual, but continued until the number present was comparatively small. There was no increase in bacteria in any cheese at any time.

The total bacteria was made up almost entirely of bacteria of the lactic class. In one pair of cheeses, at least, this included a lactic-acid-forming bacterium, liquefying gelatin very slowly and bringing about a slight proteolysis of casein in milk if the acid was neutralized.

Gelatin-liquefying bacteria were present during the first few days of the ripening in considerable numbers; after a rapid initial decline they persisted in comparatively small numbers, but in the course of time usually disappeared almost entirely. In all cases the high initial number was made up almost entirely of a coccus forming on gelatin small, round colonies with a saucer-shaped liquefaction. There was no differentiation, qualitative or quantitative, in the bacteria that could account for the marked differences which existed between the high and the low temperature cheeses.

Autodigestions of these cheeses at different periods of the ripening, made in a way that excluded the action of organisms without inhibiting the activity of the enzymes, indicated that—

1. In the fresh cheeses bacterial enzymes were present in amounts sufficient to produce only a slight increase in the amides and ammonia.

2. In the ripe cheeses bacterial enzymes were present in quantities sufficient to produce a marked increase in amides and ammonia. This was true of all the cheeses.

3. Bacterial enzymes were formed at an earlier period and in greater quantity in the high-temperature cheeses than in the cheeses ripened at a low temperature.

Certain of the liquefying bacteria were able to secrete proteolytic enzymes, which, when added to sterile water suspensions of cheese, caused a marked increase in the amides and ammonia. A lactic-acid bacterium which was able to digest neutralized milk did not secrete, under these conditions, sufficient enzyme to change appreciably the amount of soluble nitrogenous constituents of cheese extract.

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