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A STUDY OF SOME OF THE SALTS FORMED BY CASEIN AND PARACASEIN WITH ACIDS: THEIR RELATIONS TO AMERICAN CHEDDAR CHEESE.

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SUMMARY.

1. The object of the work described in this bulletin is to study the real function of acids in relation to the important changes taking place in cheese-curd during the cheddar process of cheese-making.

2. In examining cheese for hetero-caseose by extraction with dilute solution of common salt, a body was found in quantities so large as to indicate that it was some compound other than hetero-caseose.

3. In studying cheeses made with and without the use of lactic acid, the salt-soluble product was discovered to be present in very large quantities only when acid was used, being practically absent, or present in very small proportions, when no acid was used.

4. In normal cheese the salt-soluble product is always found, but in varying quantities, being largest usually in new cheese, and diminishing with age of cheese. Various conditions affect the amount in new cheese, especially those conditions influencing the amount of acid present.

5. Paracasein, carefully prepared and treated with dilute lactic acid, furnishes a product resembling, in both physical and chemical properties, the salt-soluble substance extracted from cheese.

6. Paracasein is shown to combine with an acid in at least two different proportions, forming two distinct compounds; one is the unsaturated or mono-acid salt; the other, the saturated or di-acid salt. Such compounds were prepared with lactic, acetic, hydrochloric, and sulphuric acids.

7. Casein forms salts with acids in the same manner as paracasein.

8. The unsaturated salts formed by casein and paracasein with acids are soluble in dilute solutions of sodium chloride and in 50 per ct. hot alcohol, but insoluble in water. The saturated salts are practically insoluble in water, dilute salt-solutions and 50 per ct. hot alcohol. Both forms are sparingly soluble in dilute solutions of calcium lactate and calcium carbonate.

9. The important changes taking place in cheese-curd during the process of cheddar cheese-making, such as the acquired ability to form strings on hot iron, the changes in appearance, plasticity and texture, and probably the shrinking, are due to the formation of the unsaturated paracasein lactate.

10. The ripening process in normal cheddar cheese, by which the insoluble nitrogen-compounds change into soluble forms, begins, not with paracasein as has been universally held, but with unsaturated paracasein lactate. The water-soluble nitrogen in cheese generally increases as the unsaturated paracasein lactate decreases, and apparently at the expense of the latter compound. The first step in the normal ripening process of American cheddar cheese is probably a peptic digestion of unsaturated paracasein lactate. Some of the facts presented suggest a method of proof of the commonly accepted theory of gastric digestion.

INTRODUCTION.

The chemical changes taking place during the early stages of manufacture of cheese have hitherto been little investigated or understood. This is particularly true of the kind of cheese most extensively made in the United States and Canada, commonly called American cheddar cheese. One of the first recognized changes occurring in the process of cheese manufacture is that produced by the action of rennet on milk-casein, resulting in the formation of an insoluble curd. The action of rennet, according to Hammarsten's explanation, splits milk-casein into two different compounds[1],—one, the insoluble curd, called paracasein, formed in large proportions; the other soluble, albumose-like, called whey proteid, formed in small proportions. While Hammarsten's view is still held by many, there is growing evidence[2] that the albumose-like proteid found in whey is not a result of the action of rennet, but that it can be produced by actual proteolytic digestion of casein, due to enzymes either present in the milk itself or introduced with the rennet (as pepsin or Glaessner's pseudo-pepsin). Whatever we may ultimately find to be the exact nature of rennet action on milk-casein, it is not the only factor at work during and after the formation of the insoluble curd; for there is present at least one other active process during the early stage of cheese-making that has much to do with the production of a " good curd ", judged from the practical standpoint of a cheesemaker.

One of the first operations in cheddar cheese-making is to " ripen " the milk previous to adding rennet. This is accomplished by allowing the milk to stand some time at a temperature of about 86° F. (30° C.), or the operation may be hastened by adding to the milk special cultures of acid-producing organisms. This process, commonly known as the " ripening of milk," has for some time been recognized as an acid-producing form of fermentation, that is, a fermentation brought about by the action

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[1] *Maly's Ber.*, **2**: 118 (1872); **4**: 135 (1874).

[2] Fuld. *Beiträge zur Chim. Physiol.*, **2**: 169 (1902).

of lactic-acid organisms on the milk-sugar, resulting in the formation of lactic acid. In ripening milk, the cheesemaker increases the quantity of acid in the milk, not only before adding rennet, but continuously throughout the rest of the process of manufacture.

The amount of acid in cheese-curd is roughly measured in a mechanical way by means of the so-called " hot-iron test." When a piece of curd is pressed against a hot iron and then drawn away carefully, fine, silky threads are formed, adhering to the iron. This phenomenon is closely associated with the formation of acid and the length of the strings shown by the hot-iron test is utilized as a measure of the amount of acid present and as an indication when to perform certain operations. Thus, when the curd strings on the hot iron to the length of one-eighth of an inch, the whey is removed from the curd, after which the curd is " packed " in a pile and allowed to lie until it has passed through the so-called " matting " or " breaking-down " process, when it furnishes strings an inch or more in length by the hot-iron test. When this stage is reached, the remaining steps of the manufacturing process are at once completed, such as milling, salting, and putting in molds for pressing.

While it has been generally supposed that the presence of some acid, presumably lactic acid, in the cheese-making process is, in some way or other, responsible for the most important changes taking place, such as the shrinking of the curd, the acquired ability to form strings on hot iron and the change in appearance and plasticity of curd, no one has ever been able to show in what way these changes were brought about by acids. It has been commonly supposed that the observed changes were purely physical in character and were not the result of chemical changes in the curd. In the following pages it is our purpose to consider more fully than has been done previously the real function of acids in relation to the important changes taking place in cheese-curd during the cheddar process of cheese-making.

Since Sjöquist's[3] investigation, we have learned that proteids can build salts with acids and bases, and this subject has been

3 *Skandin. Arch. f. Physiol.*, **5** : 277 (1894).

considerably developed in the last few years. Bugarszky and Liebermann[4] have shown that egg-albumin forms with hydrochloric acid a salt analogous to ammonium chloride, which they call albuminum chloride. Osborn[5] has also shown that edestin, the globulin of hempseed, is capable of combining with acids in several different proportions, forming salts having different properties. Panormoff[6] also has recently described definite compounds formed by egg-albumin with different acids. So far as we are able to learn, no similar work with milk-casein or with paracasein (milk-casein coagulated by rennet and constituting a large proportion of cheese-curd) has been done. The nearest any one has come to touching this specific line of work was in the case of Danilewsky[7], who, in his preparation, by the use of hydrochloric acid, of what he called pure casein, actually prepared a mixture of the salts of this proteid, one of which is soluble in 50 per ct. boiling alcohol, while the other is insoluble. From this behavior, he concluded that milk-casein consists of two proteids. Reference will again be made to this.

In our early work relating to the study of chemical changes taking place in cheese during the process of ripening or curing, we mixed the cheese thoroughly with sand by grinding in a mortar and completely extracted this with water at 131° F. (55° C.). The residue, insoluble in water, was then treated with a 10 per ct. solution of sodium chloride at 104° F. (40° C.) for the purpose of removing any hetero-caseose formed during the ripening process. Chittenden[8] had shown that, in a peptic digestion of casein, hetero-caseose was formed only in small amounts. When, by our extraction of fresh or partly ripened cheese with dilute salt solution, we obtained amounts of proteid, representing often as high as 40 per ct. of the total nitrogen present in the cheese, it became apparent that we were dealing with some compound other than hetero-caseose.

[4] *Pflüger's Archiv*, **72**: 51 (1898).

[5] Ann. Report of Conn. Agr. Exp. Sta. **23**: 402 (1900).

[6] *Jour. d. Russ. Phys. Chem. Gesellsch.*, **31**: 556.

[7] *Zeit. f. Physiol. Chem.*, **7**: 433 (1883).

[8] Studies in Physiol. Chem. Yale Univ. **2**: 156(1885-6).

DISCUSSION OF EXPERIMENTS.

OCCURRENCE OF THE SALT-SOLUBLE PRODUCT IN CHEESE MADE
IN THE PRESENCE OF CHLOROFORM WITH AND WITHOUT ACID.

(*1*) *Without acid.*—Cheeses were made from milk which had been previously heated to 208° F. (98° C.) for the purpose of destroying all enzymes and bacterial forms present in the milk. After cooling the milk to the temperature employed in cheese-making, chloroform was added to the milk to the extent of 4 per ct. by volume in order to prevent any bacterial activity. As the ability of the milk-casein to coagulate with rennet is impaired by heat, this property was restored, in one case, by the addition of a small amount of calcium chloride, and, in the other case, by passing a stream of carbon dioxide through the milk for half an hour. The cheeses made from the milk thus treated were left in press over night, and were then examined at once for the salt-soluble product. The determination was made as follows: an amount of the fresh cheese, weighing 25 grams, was ground with sand and extracted with several portions of distilled water at 131° F. (55° C.) until the extract amounted to 500 cc. The residue was then similarly extracted with a 5 per ct. solution of sodium chloride. The amount of nitrogen in 50 cc. of this salt extract was determined by the Kjeldahl process. Table I gives the results obtained. The figures in parenthesis indicate the serial numbers of the different cheeses.

(*2*) *With acid.*—Other cheeses were made exactly like the preceding, except that lactic acid was added to the milk to the extent of 0.2 per ct., by weight, of the milk. An examination of these cheeses, made at once after they were taken from press, gave the results stated in Table I.

TABLE I.—AMOUNT OF NITROGEN IN SALT-SOLUBLE EXTRACT OBTAINED FROM
CHEESES* MADE WITH AND WITHOUT LACTIC ACID.

	Nitrogen, expressed as percentage of total nitrogen in cheese.		Nitrogen, expressed as percentage of cheese.	
	Made with addition of calcium chloride.	Made with addition of carbon dioxide.	Made with addition of calcium chloride.	Made with addition of carbon dioxide.
(1) With acid....	27.88 (45)	29.80 (48)	0.87 (45)	0.700 (48)
" "	26.62 (46)	22.90 (51)	0.84 (46)	0.754 (51)
(2) Without acid.	2.44 (44)	5.24 (49)	0.76 (44)	0.132 (49)
" "	2.90 (47)	5 72 (50)	0.76 (47)	0 148 (50)

An examination of this table shows that the amount of nitrogen, in the form of the salt-soluble product, was increased very greatly by the action of the added lactic acid. The cheeses made without acid show small amounts of this salt-soluble substance. In the case of Nos. 44 and 47, the salt-soluble product found may possibly be attributed to the slight amount of lactic acid formed in the milk before it was heated, or it may be that neutral paracasein itself is slightly soluble in salt-solution. There is some basis for this latter supposition, for, in the case of milk taken directly from a cow and immediately etherized and made into curd, these conditions excluding all possible acid formation, there still appears to be a slight solubility of the neutral paracasein in dilute salt-solution. In the case of Nos. 49 and 50 somewhat more salt-soluble product is found than in Nos. 44 and 47. This is undoubtedly due to the effect of the carbon dioxide used, even so weak an acid probably having some limited tendency to form with para-casein a salt-soluble product.

OCCURRENCE OF THE SALT-SOLUBLE PRODUCT IN NORMAL CHEESE.

In the many normal cheddar cheeses thus far examined by us, this salt-soluble substance is always found. The amounts vary, but the factors causing the variations have not been fully studied yet. For example, the amount of milk-sugar retained in the cheese is one cause of such variations; likewise those conditions affecting the quantitative conversion of milk-sugar into lactic acid. Then, again, we have not yet obtained satisfactory control of all the details of the quantitative estimation of this salt-soluble product. Its amount varies also with the age of a cheese. In cheese fresh from press, that is, about 24 hours old, we have found that from 40 to 78 per ct. of the total nitrogen is in the form of the salt-soluble substance, and the amount diminishes more or less with advance of age in the cheese, as illustrated by the figures in Table II.

TABLE II.—AMOUNT OF SALT-SOLUBLE PRODUCT IN CHEESES AT DIFFERENT AGES.

Serial number of cheese.	Age of cheese.	Salt-soluble nitrogen, expressed as percentage of total nitrogen in cheese.	Salt-soluble nitrogen, expressed as percentage of cheese.	Water-soluble nitrogen, expressed as percentage of total nitrogen in cheese.	Water-soluble nitrogen, expressed as percentage of cheese.
54	1 day	58.7	2.08	6.78	0.240
54	1 mo.	42.4	1 58	19.30	0.718
54	3 mos.	33.4	1.31	26.02	1.020
31–A	2 weeks	40.7	1.50	15.50	0.569
31–B	" "	42.8	1.58	16.50	0.608
31–C	" "	37.4	1.38	16.10	0.584
31–D	" "	40.8	1.49	15.80	0.589
38–A	3 mos.	16.48	0.58	37.22	1.310
38–B	" "	17.60	0.63	35.48	1.270
38–C	" "	19.19	0.71	32.16	1.190
38–D	" "	21.96	0.83	29.90	1.130
38–A	9 mos.	13.43	0.54	53.24	2.140
38–B	" "	14.29	0.57	47.75	1.910
38–C	" "	18.46	0.74	45.64	1.830
38–D	" "	18.44	0.78	40 91	1.730

An examination of Table II indicates a general tendency for the amount of nitrogen present in cheese in the form of the salt-soluble product to decrease as the cheese advances in age.

An examination of the last two columns of figures shows that, as cheese increases in age, the amount of nitrogen in the form of the water-soluble product increases. The fact that the water-soluble nitrogen in cheese increases while the salt-soluble product decreases strongly suggests that this progress in proteolysis takes place at the expense of the salt-soluble product. However, we are not inclined to assert positively that such a relation exists until we have perfected to our satisfaction the method for estimating the salt-soluble product. In cheese No. 54, one of our latest experimental cheeses, our method of extraction was under better control and the figures obtained are entirely consistent with the view that proteolysis in cheese commences with the salt-soluble product. However, the main point to which we wish to

call attention in connection with Table II is that in freshly-made cheddar cheese the salt-soluble product forms a large part of the nitrogen-compounds of the cheese.

NATURE OF THE SALT-SOLUBLE PRODUCT.

(*1*) *A salt of paracasein.*—In the case of cheeses made with and without lactic acid, in the presence of chloroform, we have seen that there were large amounts of the salt-soluble product formed when lactic acid was used, and only small amounts when no acid was used. We have also seen that, in the case of normal cheddar cheeses, the salt-soluble product occurs in large proportions, increasing in quantity as the milk-sugar disappears, or, in other words, as the amount of lactic acid increases. It, therefore, appeared to us that, in this salt-soluble product, we were dealing with some compound of paracasein and lactic acid, probably a paracasein lactate.

(*2*) *Artificial preparation of paracasein lactate.*—It seemed desirable to attempt the artificial preparation of the salt-soluble product, and to ascertain if it were identical with the substance extracted by a dilute solution of sodium chloride from newly-made cheese. For the purpose of this comparison, the following experiment was undertaken in the artificial preparation of the salt-soluble product:

We coagulated about 6 liters of skim-milk by rennet at 86° F. (30° C.). The resulting curd was well washed with water, drained, and then treated for one-half hour at 122° F. (50° C.) with 3 liters of a 1 per ct. solution of lactic acid. By this treatment a turbid solution was obtained, which was filtered from the large residue remaining undissolved, through cloth first and then through absorbent cotton. This filtrate, neutralized with dilute potassium hydroxide, gave a copious precipitate, which was filtered, washed with water, and redissolved in 1 liter of 0.5 per ct. solution of lactic acid. This solution was filtered and reprecipitated by dilute alkali, and the process of redissolving, filtering, and reprecipitating was repeated twice. The resulting product was finally well washed with water, suspended in 95 per ct. alcohol for 2 days and, after removal of alcohol by filtration, was extracted 24 hours with ether and dried at 212° F. (100° C.). The substance

thus prepared was firm and leathery in texture, greatly resembling in physical properties the product prepared from the salt-extracts of cheese. To all appearances, the lactic acid had simply combined with the paracasein, forming a compound soluble in slight excess of acid and insoluble in neutral solutions.

(*3*) *Preparation of paracasein lactate obtained from cheese.*— Cheese about 3 months old was extracted with a 5 per ct. solution of sodium chloride. To 6.5 liters of this extract, lactic acid was added to the extent of 0.2 per ct. by volume, producing a flocculent precipitate that rapidly settled. The precipitate was filtered and allowed to drain in order to remove the large amount of salt adhering. The filtrate gave no further precipitate on addition of more lactic acid. The precipitate was completely soluble in 0.5 per ct. solution of lactic acid, and this acid solution gave an abundant precipitate when neutralized by dilute alkali. This neutralization precipitate was filtered, well washed with water, and then treated, in every respect, as described above in the preparation of the artificial product. In this way, 20 grams of each product were obtained.

Comparison of natural and artificial products.—The old method of comparing two products by the results of ultimate analysis, in order to establish their likeness or unlikeness, is now recognized as faulty, especially in the case of proteid bodies. Only by cleavage and by quantitative estimation of certain end-products can a reliable basis be found for determining the question of similarity of structure in different compounds.

Determinations of nitrogen, phosphorus, and ash were made in the two products. In addition, an amount of each proteid weighing 10 grams was hydrolyzed by boiling 14 hours, on a sand-bath, under a Liebig condenser, with 60 grams of water and 30 grams of sulphuric acid of sp. gr. 1.84. In the resulting liquid, ammonia and the hexon bases were determined according to the method of Kossel and Kutscher.[9] The results are given in Table III, together with similar figures obtained by Hart with casein[10] prepared directly from milk by the use of acetic acid without rennet.

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[9] *Zeit. f. Physiol. Chem.* **31** : 165 (1898).

[10] *Ibid.* **33** : 347 (1900).

TABLE III.—COMPARATIVE COMPOSITION OF NATURAL AND ARTIFICIAL
PRODUCTS.

	Nitrogen expressed as percentage of total nitrogen in form of				Nitrogen.	Phosphorus.	Ash.
	Ammonia.	Histidin.	Arginin.	Lysin.			
					Per ct.	Per ct.	Per ct.
Natural product..	7.65	2.36	7.77	2.21	15.12	0.70	1.51
Artificial product.	7.66	2.21	8.08	2.02	15.30	0.91	1.120
Casein	7.34	3.66	9.51	2.31	15.65	0.84	......

The results embodied in Table III indicate a striking similarity in structure between the salt formed by paracasein with lactic acid found naturally occurring in cheese and that artificially prepared, so far as the products of hydrolysis enable us to draw conclusions. This has reference simply to the linking of the nitrogen in the proteid as a whole.

Later, it will be seen that what we had really prepared in both cases was the saturated salt formed by paracasein with lactic acid and not the unsaturated salt; and, as we shall show, the unsaturated salt is the one soluble in dilute solution of sodium chloride. The formation of these combinations with acids probably does not involve any deep-seated rupture of the proteid molecule, as can readily be inferred from the results given in Table III with casein, which furnishes both saturated and unsaturated salts with acids. It is evident that, in the preparation of the artificial salt identical with the proteid extracted by common-salt solution from cheese, the chief, if not the only, active factor is an acid.

SALTS FORMED BY COMBINATION OF PARACASEIN WITH ACID.

Action of lactic acid used in different amounts upon paracasein.—In the manufacture of cheese, the lactic acid that combines with paracasein is formed by fermentation of milk-sugar, the lactic acid combining with the paracasein as rapidly as the acid is formed. It appeared desirable to study the action of lactic acid, used in different amounts, as it is formed in the fermentation process, upon paracasein under well-controlled conditions, and also to compare this action with the one taking place when pure

dilute lactic acid acts upon paracasein directly without any fermentation. For this purpose it was desired to secure paracasein or cheese-curd as free as possible from acid. In order to prevent all acid fermentation, very fresh, sweet milk was treated with ether. In this way it was possible nearly to eliminate the formation of paracasein compounds of lactic acid at the beginning of the experiment and have as our original working material paracasein as nearly as possible free from salt-soluble compounds. Two analyses of paracasein, thus prepared, showed the presence of 3.90 and 4.34 per ct. of the total nitrogen in the form of a salt-soluble compound. Several bottles were prepared in the following manner: We placed in each bottle 25 grams of the carefully prepared cheese-curd or paracasein, ground with sand, and added 50 cc. of water; the mixture was thoroughly shaken, and then sterilized. To this common mixture, various additions were made in the different bottles as indicated below:

Series A received 0.5 gram of sterile milk-sugar and a pure culture of lactic-acid organism.

Series B, 1 gram of sterile milk-sugar and lactic-acid organism.

Series C, no milk-sugar and organism.

Series D, 0.5 gram of pure lactic acid and no organism.

Series E, 1 gram of pure lactic acid and no organism.

Series F, 1.5 grams of pure lactic acid and no organism.

All the bottles were kept at 60° F. (15.5° C.) and analyses were made at different periods, as indicated in Table IV.

TABLE IV.—INFLUENCE OF DIFFERENT AMOUNTS OF LACTIC ACID ON PARACASEIN.

Series.	Grams of milk-sugar used.	Age of mixture when analyzed.	Presence of milk-sugar in mixture when analyzed.	No. cc. $n/10$ NaOH required to neutralize 50 cc. of water-extract.	Precipitate appeared on neutralizing water-extract.	Nitrogen, in form of salt-soluble product, expressed as percentage of total nitrogen.
A	0.5	2 weeks	Trace	3.9	Yes	30.36
A	0.5	" "	None	4.3	" .	27.11
B	1.0	" "	Considerable	5.4	"	23.31
B	1.0	" "	"	5.2	"	20.60
C	0	" "	None	1.2	No	3.52
C	0	" "	"	1.0	"	3.75
A	0.5	1 mo.	"	4.45	Yes	40.65
A	0.5	" "	"	3.9	"	28.46
B	1.0	" "	Small amt.	5.2	"	9.76
B	1.0	" "	" "	4.9	"	7.32
C	0	" "	None	0.6	No	3.52
C	0	" "	"	0.8	"	4.07
	Lactic acid.					
D	0.5	" "	....	3.4	Yes	44.72
E	1.0	3 mos.	....	6.4	"	2.17
F	1.5	1 mo.	....	11.4	"	1.62

Attention is called to certain facts shown by the data embodied in this table.

1. When we used 0.5 gram of milk-sugar, the maximum amount of the product soluble in dilute solution of sodium chloride was found only after the milk-sugar had completely disappeared, as can be seen by comparing Series A at 2 weeks and 1 month.

2. When we used 1 gram of milk-sugar, the largest amount of salt-soluble product was formed, while considerable sugar was still present. As the acid fermentation of the milk-sugar continued, the amount of salt-soluble product rapidly decreased. This is shown by comparing Series B at 2 weeks and 1 month.

3. We see the same truth illustrated in Series D, E, and F, where we added pure lactic acid directly in the different quantities indicated. When we added 0.5 gram of lactic acid, there was formed a large amount of salt-soluble product, nearly 45 per ct. of the total nitrogen appearing in this form. However, when we used 1 and 1.5 grams of pure lactic acid, there was practically no salt-soluble product formed.

4. Even in the cases where the largest amounts of lactic acid were used, the acid disappeared as free acid. This was shown by making a titration of the water-extract of the curd with $\frac{n}{10}$ sodium hydroxide, using phenol-phthalein as indicator. It is readily seen that the number of cc. of alkali required for neutralization represents a very small amount of acid, compared with that furnished in Series A, B, D, E, and F. The acid had largely combined with paracasein to form products only slightly soluble in water.

The question may be raised here as to what it is in the water-extract that neutralizes alkali. In Series C it is, in all probability, direct neutralization by proteid, as no acid was present. In Series F, on the other hand, there is probably some free acid, as an excess of acid was used. In most cases, however, the neutralization is due, apparently, to a water-soluble compound of acid and proteid, as shown by the fact that only in cases where acid was furnished, whether by fermentation or by direct addition, a precipitate appeared on neutralization, and such a precipitate would be expected only in the case of a combination of acid with proteid. The larger the amount of alkali required by the water-extract for neutralization, the more abundant was the precipitate appearing on neutralization.

Just here, the main point we desire to consider is an explanation of the fact to which attention has been called above, viz.: that from a given amount of paracasein treated with a certain amount of lactic acid, we obtain a maximum yield of our salt-soluble product; while an increase of acid beyond a given amount decreases the yield of salt-soluble product. We might naturally expect that increase of acid would yield an increase of salt-soluble product from a given quantity of paracasein. How can we explain this? The explanation that agrees most satisfactorily with the experimental data at hand is this: paracasein combines with lactic acid in at least two different proportions, forming two distinct compounds. One of these compounds is the unsaturated salt and is soluble in dilute solutions of sodium chloride; it is this compound that is present in normal cheddar cheese. The other compound is the saturated salt formed by the combination

·of lactic acid with paracasein and is insoluble in dilute salt solution.

(*2*) *Salts formed by combination of paracasein with other acids.*—Paracasein forms salts also with other acids. Mr. A. J. Patten has determined for us the amounts of acetic, hydrochloric, and sulphuric acids required to form the unsaturated and saturated salts. In each case, acid was added to 25 grams of fresh curd, previously ground with sand, suspended in water, and finally extracted at 122° F. (55° C.) with a 5 per ct. solution of sodium chloride.

TABLE V.—FORMATION OF COMPOUNDS BY PARACASEIN AND DIFFERENT ACIDS.

	Acetic acid.		Hydrochloric acid.			Sulphuric acid.		
·Grams of acid used	0.5	1.0	0.15	0.25	0 50	0.15	0.25	0 50
Percentage of total nitrogen found in form of salt-soluble compound	70 0	5.3	27.3	53.0	5.7	19 5	41.5	2.4

In this table the same general results are seen as in the case of lactic acid. A certain amount of acid forms with a given amount of paracasein a maximum quantity of salt-soluble product, and increase of acid beyond this quantity forms a compound not soluble in dilute salt-solution.

It also appears from the results given in Table V that mineral acids are required in smaller amounts than organic acids to saturate paracasein completely.

SALTS FORMED BY COMBINATION OF CASEIN WITH ACIDS.

(*1*) *Formation of unsaturated casein salt.*—We can show that casein readily forms an unsaturated and a saturated salt with an acid. In preparing the unsaturated salt, we used 5 cc. of freshly drawn milk, diluted it with 50 cc. of water, warmed the solution to 104° F. (40° C.), and then added $\frac{n}{10}$ hydrochloric acid until the resulting precipitate settled with a clear supernatant liquid, which required 2.7 cc. To this mixture we added 2.5 grams of sodium chloride, thus making a 5 per ct. salt-solution. On warm-

ing, the unsaturated casein chloride completely dissolved. The experiment was repeated several times with a uniform result. On adding more acid to the salt-solution, the proteid reprecipitated as the saturated chloride of casein. Casein behaved toward acetic acid in the same manner.

(2) *Determination of amount of* $\frac{n}{10}$ *hydrochloric acid required to saturate casein.*—Fresh milk was drawn from a cow directly into a bottle containing ether, thus checking all acid fermentation. We diluted 5 cc. of this milk, containing .0261 grams of nitrogen, with 50 cc. of water that had been boiled free from all carbon dioxide. We warmed the mixture to 104° F. (40° C.) and added 10 cc. of $\frac{n}{10}$ hydrochloric acid. It was desired now to determine the amount of hydrochloric acid that had not combined with casein. Determinations of the uncombined acid did not give uniform results, when the titration was made directly after the addition of acid. We therefore employed the method of using calcium picrate.[11] We added 5 cc. of a neutral solution of calcium picrate to precipitate all proteids completely. The precipitate was filtered, and the filtrate titrated with $\frac{n}{10}$ sodium hydroxide, using rosolic acid as an indicator. The reactions taking place can be represented, theoretically, as follows:

Casein $(OH)_2+3HCl$ (excess)$=$Casein Cl_2+2H_2O+HCl.

Casein Cl_2+Ca picrate $+ HCl=$Casein picrate $+ CaCl_2+HCl$.
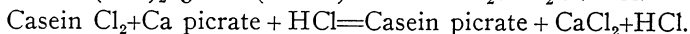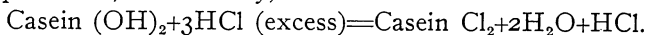
When calcium picrate is used, the chlorine which was combined with casein is changed into calcium chloride and, in this form, does not interfere with titration by alkali. Thus, we are able to determine more satisfactorily the amount of acid left uncombined with proteid.

In the experiment employed, it required 7.4 cc. of $\frac{n}{10}$ sodium hydroxide to neutralize the excess of acid, indicating that 2.6 cc. of acid had entered into combination with .0261 grams of proteid nitrogen. Using 6.39 as the nitrogen factor of casein, .0261 gram of nitrogen is equivalent to 0.167 gram of casein, and this amount combined with 2.6 cc. of $\frac{n}{10}$ hydrochloric acid, or 1 gram of casein would require for complete saturation 15.6 cc. of $\frac{n}{10}$

[11] Rohrer. *Archiv f. Physiol.*, **90**: 368 (1902)

hydrochloric acid. The work, repeated on 5 cc. of milk containing .031 gram of nitrogen, gave 15.9 cc. of $\frac{n}{10}$ hydrochloric acid as the amount required to saturate 1 gram of casein.

In the manner in which this work was done, it is recognized that we were dealing not with casein alone, but with the two principal proteids of milk, casein and albumin. However, there is no reason to believe that the results would be essentially different with pure casein. We shall later secure results with casein alone.

(3) *Determination of amount of $\frac{n}{10}$ hydrochloric acid required to saturate the unsaturated salt of paracasein.*—To 50 cc. of the salt-extract of some curd, containing .058 gram of nitrogen in the form of the unsaturated hydrochloric acid salt of paracasein, we added 10 cc. of $\frac{n}{10}$ hydrochloric acid for the purpose of converting the unsaturated into the saturated salt. We then added 5 cc. of neutral calcium picrate to facilitate clear filtration. The mixture was filtered, the precipitate washed, and the filtrate treated with $\frac{n}{10}$ sodium hydroxide, rosolic acid being used as indicator. Neutralization required 7.1 cc. of $\frac{n}{10}$ sodium hydroxide. Hence, to saturate the salt-soluble compound, equivalent to .058 gram of nitrogen, required 2.9 cc. $\frac{n}{10}$ hydrochloric acid. Using 6.39 as the nitrogen factor of paracasein, it required 7.83 cc. of $\frac{n}{10}$ hydrochloric acid to convert 1 gram of the unsaturated into the saturated hydrochloric acid salt of paracasein.

We saw above that it required from 15.6 to 15.9 cc. $\frac{n}{10}$ hydrochloric acid to saturate milk-casein completely, or, more strictly, casein mixed with a small amount of milk-albumin. Now, the amount of $\frac{n}{10}$ hydrochloric acid required to convert the unsaturated into the saturated hydrochloric acid salt of paracasein, 7.83 cc., is just one-half of 15.7 cc., the amount of $\frac{n}{10}$ hydrochloric acid required to form a saturated compound. Hence, the amount of combined acid in the saturated salt is twice that in the unsaturated salt; in other words, the unsaturated salt appears to be a mono-acid salt, and the saturated a di-acid salt. These results, it is true, were obtained, in one case, with milk-casein not strictly pure and, in the other case, with the paracasein deriva-

tive of milk-casein, and it may be thought that results secured in this way do not justify the conclusion drawn in regard to the existence of two such distinct combinations of an acid with casein and paracasein. But, aside from the data presented here, there is abundant evidence that both milk-casein and paracasein form at least two salts, corresponding to a half-saturated or mono-acid salt, and a saturated or di-acid salt.

POSSIBILITY OF AN ADDITIONAL COMPOUND OF PARACASEIN WITH ACID.

· There is evidence that small amounts of acid can disappear in cheese-curd without a corresponding increase of salt-soluble compound. This fact is illustrated by the following experiment: We coagulated with rennet 3 liters of freshly drawn milk, to which ether had been promptly added, and then warmed the milk to 107° F. (42° C.) in order to shrink and harden the curd. Portions of 25 grams each of this curd were ground with sand, placed in Erlenmeyer flasks, covered with 100 cc. of water, heated for 10 minutes at 185° F. (85° C.) to check completely all acid fermentation, and then treated with different amounts of lactic acid. After standing one hour, with occasional shaking, the curd in the different flasks was extracted, first with small amounts of water and then with 500 cc. of 5 per ct. solution of sodium chloride. In 50 cc. of each of these extracts, the nitrogen was determined by the Kjeldahl method. The results are given in the following table:

TABLE VI.—AMOUNTS OF SALT-SOLUBLE COMPOUND FORMED BY DIFFERENT AMOUNTS OF ACID.

Grams of lactic acid used................	0	0.02	0.05	0.10	0.20	0.50
Percentage of total nitrogen in form of salt-soluble compound................	6.0	7.0	8.1	8.6	23.0	72.0

The amount of salt-soluble compound was not proportionately increased by the use of .02, .05 and .10 gram of lactic acid for 25 grams of curd; but when an additional .10 gram of lactic acid

was used, bringing the total up .20 gram of lactic acid, there was a large increase of the salt-soluble compound, and this was still greatly increased by additions of lactic acid above .20 gram up to .50 gram. It is possible that there may be formed with acids a paracasein salt containing still less acid than the half-saturated salt, or, it may be that the acid which disappears unites with some of the insoluble inorganic salts in the curd.

SOLUBILITIES OF THE SALTS FORMED BY CASEIN AND PARACASEIN WITH ACIDS.

(*1*) *Saturated salts.*—The saturated acetic and lactic acid salts of casein and paracasein, as well as the hydrochloric and sulphuric acid salts, are practically insoluble in all the media tried. They are insoluble in water; in 5 and 10 per ct. solutions of sodium chloride; slightly soluble in 50 per ct. boiling alcohol, practically insoluble at 122° F. (50° C.) in saturated solution of calcium carbonate, and in 2 per ct. solution of calcium lactate at 122° F. (50° C.). They dissolve, of course, in excess of acid or dilute alkali.

(*2*) *Unsaturated salts.*—The unsaturated or half-saturated salt of each of the acids mentioned above is insoluble in water; completely soluble in 5 per ct. solutions of sodium chloride at 130° to 140° F. (55° to 60° C.), not separating again on cooling, but entirely separating by dialysis in water; soluble in 50 per ct. boiling alcohol, but separating again on cooling; only slightly soluble in saturated solution of calcium carbonate at 122° F. (50° C.), or in 2 per ct. solution of calcium lactate.

(*3*) *Explanation of Danilewsky's error.*—Danilewsky's[12] early supposition, that casein consists of two proteid bodies separable by hot 50 per ct. alcohol, finds a reasonable explanation in the fact that the unsaturated salt formed by casein with an acid is soluble in 50 per ct. hot alcohol, while the saturated salt is insoluble in this medium. When the usual method is employed of precipitating casein from milk by means of dilute acid, the resulting product can easily be a mixture of the unsaturated and satu-

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[12] *Zeit. f. Physiol. Chem.*, **7**: 227 (1883).

rated salts, the quantity of each present depending upon the amount of acid used in the precipitation. Danilewsky, in his work, used only enough hydrochloric acid to effect a good precipitation of milk-casein and thus he formed more or less of the unsaturated salt, soluble in hot 50 per ct. alcohol. Thus, it is easy to understand how he came to the erroneous conclusion that milk-casein consists of two proteids.

(4) *Relation to Freudenreich's work.*—It will be noticed that the solubility of the two salts in solution of calcium carbonate or calcium lactate is slight. In the case of the saturated paracasein salt, it was found in one experiment that only 1.2 per ct. of the salt was soluble in saturated solution of calcium carbonate at 122° F. (50° C.) and only 1.12 per ct. was soluble in a 2 per ct. solution of calcium lactate at 122° F. (50° C.). The unsaturated paracasein salt had a solubility of 5.01 per ct. in calcium carbonate solution and 4.70 per ct. in the calcium lactate solution.

The determination of these solubilities was made in the hope of finding some explanation of the results obtained by Freudenreich[13] in his studies relating to the power of lactic-acid organisms in effecting proteolytic changes in milk-casein. Freudenreich found that sterile milk, treated with powdered calcium carbonate and then seeded with certain lactic-acid-producing bacteria, showed an increase of soluble nitrogen-compounds, in both caseoses and amides. He determined the soluble nitrogen by passing the milk through a Chamberland filter. He does not state the amount of total nitrogen in the milk at the beginning of the experiment but only the amount of soluble nitrogen. Assuming that the milk used by Freudenreich contained 0.5 per ct. of total nitrogen, the average of milk, and recalculating his results in percentages of total nitrogen, we obtain the data given in the following table:

[13] *Landw. Jahr. d. Schweiz*, **12**: 279 (1898).

TABLE VIII.—RESULTS OF FREUDENREICH CALCULATED ON BASIS OF TOTAL NITROGEN IN MILK.

Age of milk when analyzed.	Soluble nitrogen, expressed as percentage of total nitrogen in milk.	Amide nitrogen, expressed as percentage of total nitrogen in milk.	Organism used.
Beginning of experiment.	6.6	......	....
9 months................	47.0	34.6	Milk culture Bac. ε from rennet.
13 "	44.4	30.2	" " " ε " cheese.
13 "	49.2	......	" " " ε " "
14 "	42.8	28.0	Bac. α and Bac. ς.
9 "	18.8	10.6	" α.

The amounts of soluble nitrogen reported by Freudenreich as being formed by the action of different lactic-acid-producing organisms are considerable. Moreover, it is to be noticed that quite large amounts of amide nitrogen were formed. While there were in the flasks used solutions of calcium carbonate and of calcium lactate, we can not explain the relatively large amounts of soluble nitrogen by the solubility of either the saturated or unsaturated lactates of casein in solution of calcium carbonate or lactate. Whether his explanation is correct or another is to be found, Freudenreich's results clearly represent actual proteolytic changes of casein.

Nicholson[14] repeated Freudenreich's work, using the common lactic acid bacterium found in cheese, but failed to find any proteolysis of casein. Nicholson says: " In some cases, the table shows a slight increase in soluble nitrogen, where the calcium carbonate has been added; in others, there is no increase. In the uncarbonated milk there is absolutely no increase in any case." The slight increase reported by Nicholson in the case of the milk containing calcium carbonate can readily be explained on the basis of the slight solubility of the lactic-acid salts of casein in solution of calcium carbonate or calcium lactate.

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[14] Thesis for Master's Degree—Univ. of Wis. 1902.

We have recalculated a portion of Nicholson's results on the same basis as that used with Freudenreich's figures, and give them in the table following.

TABLE VIII.—RESULTS OF NICHOLSON'S WORK WITH LACTIC ACID BACTERIA.

Without calcium carbonate.			With calcium carbonate.		
Age of milk when analyzed.	Soluble nitrogen, expressed as percentage of total nitrogen in milk.	Amide nitrogen, expressed as percentage of total nitrogen in milk.	Age of milk when analyzed.	Soluble nitrogen, expressed as percentage of total nitrogen in milk.	Amide nitrogen, expressed as percentage of total nitrogen in milk.
0	8.8	3 8	0	9.6	4.0
0	9.2	3.6	0	9.8	4.0
59 days	13.2	3.2	28 days	18.0	4.0
59 "	12.6	3.0	28 "	21.8	2.8
450 "	10.2	1.4	257 "	12.6	2.2
450 "	8.4	trace	257 "	12.2	trace
....	....	....	314 "	13.2	2 8
....	....	....	314 "	12.0	3.2
....	....	....	450 "	12.6	trace
....	....	....	450 "	14.0	trace

In some of our future work bearing more directly upon the chemical changes taking place during the process of cheese-ripening, we shall have occasion to refer to this subject again.

RELATION OF THE UNSATURATED PARACASEIN LACTATE TO THE CHEDDAR PROCESS OF CHEESE-MAKING.

As previously stated, it has been supposed that the presence of lactic acid was, in some way or other, responsible for, or associated with, some of the most important changes taking place in the curd during the cheese-making process, such as the acquired ability to form strings on a hot iron and the change in appearance and plasticity of curd; but no one has previously been able to show beyond question that these changes were actually brought about by an acid, or in what way they were accomplished. We purpose now to show the intimate relation existing between the presence of unsaturated paracasein lactate and some of the important changes occurring in the curd during the process of making cheese.

(*1*) *Property of curd to string on hot iron due to unsaturated casein lactate.*—We coagulated by rennet about 27 liters of milk, properly ripened for cheese-making, and carried on the process of treatment in the usual normal manner employed in manufacture. At different intervals, samples of curd were taken in order to determine the amount of salt-soluble compound present; also, samples of whey were taken at the same time for the purpose of determining the amount of milk-sugar. The changes took place rather slowly in this instance, owing to the fact that the milk was more than usually sweet for cheese-making purposes; that is, it was so fresh as to contain only small amounts of lactic acid at the start.

We present here our results in tabular form.

TABLE IX.—INCREASE OF SALT-SOLUBLE COMPOUND IN CURD WITH DECREASE OF MILK-SUGAR IN WHEY.

Time of taking sample of curd.	Percentage of total nitrogen in form of salt-soluble compound.	Percentage of milk-sugar in whey.	Remarks.
Rennet added at 9.30 a. m.			
10 o'clock a. m..	5.0	4.75	Curd did not string.
12 " m	6.1	....	Curd just began to string. Whey removed from curd.
4 " p. m..	31.7	1.83	Curd formed strings 1 inch long. Curd put in press.

The results in this table show that as the amount of salt-soluble compound present in the curd increased, the curd acquired the property of forming longer strings on the hot iron. That this was due to the increase of salt-soluble compound is confirmed by the fact that the unsaturated salt of casein lactate itself, when isolated from curd or cheese, forms on hot iron beautiful, fine, silky threads, of almost any length desired. The same is true also of the product artificially prepared.

(*2*) *"Breaking-down" process in curd due to formation of unsaturated paracasein lactate.*—After the whey is removed from the curd in the process of cheese-making, the curd is "packed", or

" matted ", that is, piled in a heap, and kept in this condition, with occasional repacking, until it has gone through the regular " breaking-down " process, in the course of which the curd undergoes several marked, easily discernible changes in physical properties. From a tough, rubber-like consistency, with a high water-content, the curd changes to a mass having a smooth, velvety appearance and feeling, and a softer, somewhat plastic consistency. The texture also changes so that the curd acquires a peculiar kind of grain and tears off somewhat like the cooked meat of a chicken's breast.

These marked changes in the physical properties of the curd can be most readily and satisfactorily explained by attributing them to the increasing quantity of the unsaturated casein lactate produced throughout the cheese mass, as the result of continuous formation of lactic acid by the fermentation of the milk-sugar present. These changes in the properties of curd take place simultaneously with the formation of unsaturated casein lactate and do not take place in the absence of this compound. The softening of curd, or " breaking-down " process, is attributed by Duclaux to the action of vibrios, but this is certainly an error so far as relates to American cheddar cheese.

(3) *Abnormal softening of curd on addition of salt, due to the presence of the unsaturated paracasein lactate.*—It sometimes happens that, when curd is salted, it softens remarkably, appears to be slimy, and slips through the fingers when squeezed in the hand. This is a condition which has not been satisfactorily explained; the usual explanation is that liquefying organisms have prevailed over all others and have dissolved some of the curd. The real explanation is as follows: When curd behaves, on salting, in the manner described above, more than the usual amount of lactic acid has been formed and, hence, more than the usual proportion of unsaturated paracasein lactate has resulted. When salt is added to such curd, we at once have a brine formed, which is capable of dissolving the unsaturated paracasein lactate contained in the curd, thus producing a pasty, soft, slippery mass.

In an experiment in which we made cheese from pasteurized milk, we endeavored to simulate the action of lactic acid, as

formed by fermentation in ordinary cheese-making, by substituting in its place hydrochloric acid, added in small quantities from time to time, both before and after the addition of rennet. Paracasein chloride was readily formed by the curd, but the use of somewhat too much acid produced an abnormally large proportion of the unsaturated salt, which, on addition of common salt, formed a mushy, slippery mass. The curd, in this condition, was suspended in water and more hydrochloric acid was added, resulting in the production of a firm, exceedingly hard curd, due to the conversion of unsaturated into saturated paracasein chloride, the latter compound not being soluble in salt-solution.

(*4*) *Amount of unsaturated paracasein lactate in newly-made cheese.*—In new cheese, fresh from press, prepared under normal conditions, we have found as much as 78.5 per ct. of the nitrogen-compounds present in the form of unsaturated paracasein lactate. The proportions of this compound in new cheese depend upon a variety of conditions, which we are engaged in studying. It appears probable that, in the case of normal, newly-made cheese, the nitrogen-compounds consist mainly of unchanged paracasein and unsaturated paracasein lactate, the latter compound being present in larger proportions. Saturated paracasein lactate probably does not occur in normal cheese, but only in cheese made with excessive acid, as, for instance, cheese made from sour milk.

RELATION OF UNSATURATED PARACASEIN LACTATE TO CHEESE-RIPENING.

As cheese ages, it undergoes various changes, passing from an insoluble and flavorless substance into the palatable, more easily digestible, and nutritious material we use as food. The term " ripening " is applied to the general process that embraces these changes. Among the most extensive and profound changes occurring in cheese-ripening are those experienced by the nitrogen-compounds of the newly-made cheese, chief of which is unsaturated paracasein lactate.

A large amount of bacteriological and chemical work has been done in order to ascertain the cause of the changes that occur in

the nitrogen-compounds during the ripening of cheese. All of this work has assumed that paracasein formed the primary point of attack or starting point in these changes. Our results, presented in the foregoing pages, suggest that unsaturated paracasein lactate, and not paracasein, is the real material with which the process of cheese-ripening commences. It is not our purpose, at this time, to go into the subject in more than a preliminary way. In connection with Table II, p. 60, attention has been called to the fact that the amount of water-soluble nitrogen in cheese, which is a general measure of the extent of cheese-ripening, increases at the same time that the unsaturated paracasein lactate decreases, and probably at the expense of the latter compound.

The belief that the proteolytic changes of cheese-ripening actually start with the unsaturated paracasein lactate receives confirmatory evidence from the fact that, in the absence of unsaturated paracasein lactate in cheese, we have very incomplete proteolytic change. In order to establish beyond question the specific relation of unsaturated paracasein lactate to the proteolytic changes of cheese-ripening, we have under way numerous experiments in which preparations of pure unsaturated paracasein lactate are being subjected to the action of a variety of agencies, such as different enzymes and organisms that have been commonly associated with the different theories of cheese-ripening.

Some results, already secured, appear to indicate that the first proteolytic changes in cheese-ripening are the result of peptic digestion of unsaturated paracasein lactate. In cheese, pepsin appears to act, not to any marked extent upon the proteid paracasein, but only upon its unsaturated compounds with acids. Hence, the function of an acid in cheese-making is the production of the unsaturated compound of paracasein with acid. This suggests that something of the same kind of action may be found to be true in the case of gastric digestion. It is well established that pepsin acts upon proteids in the stomach only in the presence of dilute acid. The function of the hydrochloric acid of the gastric juice, like lactic acid in cheese, may be the formation of compounds with proteids; in other words, it may be found that pepsin

in gastric digestion does not act upon pure proteids but only upon their compounds with acids, in this case hydrochloric acid. While this general view has been held for some time, there have not, so far as we know, been any actual experiments previously made in testing the action of pepsin upon pure compounds formed by proteids with acids. We have done this only in the case of the unsaturated paracasein lactate, but this appears as the first instance to furnish proof in an individual case of what may be found true, in general, of all cases of peptic digestion.