SOME OF THE RELATIONS OF CASEIN AND PARA-CASEIN TO BASES AND ACIDS, AND THEIR APPLICATION TO CHEDDAR CHEESE.

L. L. VAN SLYKE AND E. B. HART.

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SOME OF THE RELATIONS OF CASEIN AND PARACASEIN TO BASES AND ACIDS, AND THEIR APPLICATION TO CHEDDAR CHEESE.

L. L. VAN SLyKE AND E. B. HART.

SUMMARY.

1. Object.—The primary object of the work presented is to make a further study of the compounds first discussed in Bulletin No. 214, casein and paracasein salts of acids, with an extension to the free proteids and some of their combinations with bases.

2. Base-Free Casein.—Preparations of casein free from ash or nearly so were made by precipitating dilute skim-milk with acids, removing the acid and inorganic matter by repeated filtration and trituration with water in a mortar. The process required several days.

3. Basic Calcium Casein.—Preparations were made with base-free casein in which the proteid combined with about 2.40 per ct. of calcium oxide. One such preparation was made by triturating together calcium carbonate and the base-free casein suspended in water, and another by dissolving base-free casein in lime-water and making this neutral to phenolphthalein by acid.

4. Neutral Calcium Casein.—By treating base-free casein dissolved in lime-water with acid until the reaction is almost neutral to litmus, there is formed a compound of casein and calcium oxide containing about 1.50 per ct. of calcium oxide.

5. Calcium Casein Compounds in Relation to Ren-
net Enzym and Soluble Calcium Salts.—Rennet enzyme coagulates neither neutral nor basic calcium casein. Neutral calcium casein after treatment with rennet is coagulated at ordinary temperatures by soluble calcium salts. Soluble calcium salts, as calcium chloride, coagulate both neutral and basic calcium casein on warming to 35° to 45° C.

6. Casein Present in Milk as Neutral Calcium Casein.—In its behavior toward soluble lime salts on warming and at ordinary temperatures after treatment with rennet, neutral calcium casein behaves like milk-casein, and casein is probably present in cows' milk as the neutral calcium casein.

7. Identity of Base-Free Casein and the Salt-Soluble Proteid.—A base-free casein, prepared either directly from milk or by treating a lime-water solution of free casein with an acid to the point of acidity with litmus, is readily soluble in warm 5 per ct. salt solution and in hot 50 per ct. alcohol. This body, when freshly prepared and sufficiently warmed is very plastic and ductile. It behaves in all respects like the compound which we were formerly led to regard as a compound formed by combination of casein and an acid and which we regarded as a casein mono-salt of the acid precipitant.

8. Relation between Two Series of Compounds previously called Casein Mono-Salts and Casein Di-Salts of Acids.—When one gram of base-free casein is treated with about .5 cc. of \( \frac{v}{10} \) hydrochloric acid, a substance is formed which is insoluble in warm 5 per ct. salt solution and in hot 50 per ct. alcohol and which is no longer plastic or ductile on warming. This is like the substance usually formed when milk coagulates by natural souring. By treating base-free casein with dilute acid, it was found that one gram of base-free casein appears to combine with about .5 cc. of \( \frac{v}{10} \) hydrochloric acid, forming a casein salt of hydrochloric acid. While the compounds formerly regarded by us as
casein mono-salts of acids have been shown by us to be identical with base-free casein, the compounds which we called casein di-salts of acids are compounds formed by combination of acids with free casein.

9. *Paracasein and Its Compounds.*—A preparation of base-free paracasein was made, and from this dissolved in lime-water were prepared (1) basic calcium paracasein, containing in combination about 2.40 per ct. of calcium oxide, and (2) neutral calcium paracasein containing about 1.50 per ct. of combined calcium oxide.

10. *Comparison of Properties of Casein and Paracasein and their compounds of Calcium.*—Basic calcium casein and paracasein appear soluble in water forming slightly opalescent solutions. Neither is coagulated by rennet, but both are precipitated by soluble calcium salts on warming. Neutral calcium casein is coagulated by soluble calcium salts on warming to 35° to 40° C, but not at ordinary room temperature while neutral calcium paracasein is completely and quickly coagulated at room temperatures by soluble calcium salts. Free casein and free paracasein, freshly prepared, possess the same solubilities in warm 5 per ct. salt solution and in hot 50 per ct. alcohol; they also possess the same peculiar properties of plasticity and ductility. The close resemblance of casein and its compounds respectively to paracasein and its compounds suggests that they are chemically alike, paracasein being different only by consisting of a larger molecular aggregation than casein.

11. *Relation of Paracasein to Salt-Soluble Substance and to Body formed by Treatment with Acid.*—Free paracasein appears to be identical in characteristic properties with the compounds we formerly called paracasein mono-salts of acids used as precipitants. The compounds which we have heretofore called paracasein di-salts of acids appear to be combinations of free paracasein and acids used as precipitants, one gram of paracasein uniting, for example, with about .5 cc. of decinormal hydrochloric acid.

12. *Relation of Salt-Soluble Product of Cheese to Paracasein.*—From water-extracted fresh cheddar cheese we prepared one extract by warm 5 per ct. salt
solution and another by hot 50 per ct. alcohol. These preparations have in common with free paracasein the characteristic properties of plasticity, ductility, and the same combining power with bases and acids, and therefore appear to be free paracasein instead of paracasein mono-lactate as we were formerly led to believe.

13. **Chemical Changes in Calcium Casein resulting from Sourcing of Milk or Addition of Acids.**—When an acid is formed in or added to cows’ milk, the acid first combines with the bases of some of the inorganic salts of the milk and then with the calcium that is combined with the casein, resulting in the formation of a precipitate which is free casein. By further formation or addition of acid, the free casein unites with acid, forming a casein salt of the acid, this compound, in the case of lactic acid, being the coagulum familiar in the ordinary souring of milk.

14. **Chemical Changes in Calcium Paracasein during the Process of making Cheddar Cheese.**—The coagulum, following the addition of rennet enzym to milk is calcium paracasein, either mixed or loosely combined with soluble calcium salts. While lactic acid is being formed in the process of cheese-making, it combines with the calcium of the calcium paracasein, forming free paracasein and calcium lactate. It is this free paracasein thus formed that is soluble in warm 5 per ct. salt solution and in hot 50 per ct. alcohol and possesses characteristic properties of plasticity and ductility.

15. **Suggestions in regard to the Nomenclature of Casein and Paracasein and Their Compounds.**—Much confusion prevails at present in the use of the terms casein and paracasein. It is suggested that the following nomenclature be used: (1) That the compound existing in cows’ milk be called calcium casein. (2) That only the free proteid be called casein. (3) That the casein compound containing 2.40 per ct. of calcium oxide be called basic calcium casein. (4) That a compound formed by precipitation and combination with an acid be called a casein salt of the acid used. (5) That the same nomenclature be applied to the corresponding paracasein bodies, with the following addition: Calcium paracasein should be applied to the uncoagulated form and the term coagulated calcium paracasein to the coagulated form.
INTRODUCTION.

For several years we have been studying the chemical changes that occur in the process of cheese-ripening. Early in our work we extracted fresh and partially ripened cheese with dilute solution of sodium chloride, following a suggestion of Chittenden, who had shown that, in a peptic digestion of casein, heterocaseose was formed only in small amounts. Since we obtained by our extraction with dilute salt solution amounts of proteid representing often as much as 78 per ct. of the total nitrogen present in the cheese, it became apparent that we were dealing with some compound other than heterocaseose. The first suggestion throwing any light on the method of formation of this salt-soluble substance came in the following manner: In studying the action of galactase in cheese-ripening, we made cheddar cheese from milk containing chloroform, added for the purpose of inhibiting bacterial action. Under these conditions no lactic acid was present. In order to approximate more closely the conditions present in normal cheddar cheese, we added in a parallel experiment a small quantity of lactic acid, the other conditions of experiment being kept uniform. We noticed at once that when no acid was present in the cheese, we found little or no salt-soluble substance; while in the cheese containing a little added lactic acid there were present marked quantities of the salt-soluble product. This fact suggested that lactic acid was a necessary agent in forming the salt-soluble proteid found by us in cheese. Working along the line furnished by this clue, we were led to believe that there is a chemical combination between the paracasein of fresh cheese-curd and the lactic acid formed from milk-sugar during the cheese-making process. We were able to form salt-soluble proteids also by treating paracasein with such acids as acetic, hydrochloric and sulphuric. In each case definite amounts of acid disappeared and the proteid treated with acid underwent marked and definite changes in properties. By treating the salt-soluble compound with about the amount of acid required to convert paracasein

1Studies in Physiol. Chem., Yale Univ., 2: 156 (1885-6).
into the salt-soluble substance, we obtained a body insoluble in
salt solutions and differing also in other properties. We were
thus led to conclude that two sets of salts were formed by treat-
ing paracasein with acids, one containing twice as much acid in
combination as the other. We therefore called the salt-soluble
compound found in normal cheese paracasein monolactate, and
the one insoluble in salt solution, paracasein dilactate. We also
found the same behavior in the case of milk-casein when treated
by acids. The two series of compounds varied in respect to many
of their properties. The details of this work were described in
Bulletin No. 214. Our conclusions were based upon the fact
that when paracasein or casein is treated with an acid in definite
quantities, we obtain compounds of definitely characteristic prop-
erties.

We desired, however, to carry our work farther, if possible, and
prove beyond question that there is an actual chemical combi-
nation between acid and proteid. We desired to prepare the free
proteid and then treat this with acid and study the results.

Hammarsten made a special study of the question of com-
bination between casein and acids, and concluded that there was
no ground for believing that a chemical combination takes place
between casein and the acid used to precipitate it. He based his
statement on the fact, that, by rubbing for several days in a
mortar with different portions of water a precipitate formed by
casein with an acid, he was able to remove the acid so completely
that the remaining precipitate gave no test for acid. The sub-
stance which he used in his work was what we have called the
di-acid compound of casein. In repeating Hammarsten's work,
we were able to remove all traces of acid from the acid-precipi-
tated casein by the same treatment. We were able by this pro-
cess to prepare a substance free from the acids used as precipi-
tants and also practically free from ash. We found this freshly
prepared substance readily soluble in warm dilute salt solution
and also in hot dilute alcohol; it also showed the ductile and
plastic properties given by the substance we called a mono-salt of
casein. The facts as they presented themselves to us at this
point were as follows: A precipitate formed by treating milk-

\footnote{Maly Jahresber. d. Thierchem., 7: 160 (1877).}
casein with an acid, being what we called a di-salt of casein, was by continuous washing and trituration converted into a substance free from the acid used as precipitant and nearly free from ash; the substance precipitated by acid, insoluble in dilute salt-solution and in hot 50 per ct. alcohol, was by continuous washing and rubbing converted into a substance easily soluble in warm dilute salt solution and in hot 50 per ct. alcohol. There was also a simultaneous change in other properties. In other words, what we called a di-salt of casein was changed into what we had called mono-salt of casein, but the latter instead of being combined with an acid was free from acid and ash.

In our early study we recognized in a limited way the relation of inorganic salts in milk to the neutralization of acid. In Bulletin, No. 214, p. 428, we noticed that when milk, coagulated by rennet, is treated with varying amounts of acid, the amount of salt-soluble substance did not increase until a certain amount of acid had been used, after which it increased quite out of proportion to the increased amount of acid used. It was suggested that the formation of the salt-soluble compound did not begin until certain inorganic salts of the milk had been neutralized.

After developing the later facts described above, we proposed to study more fully the following questions:

1. What is the relation between milk-casein and the salt-soluble compound which we called a casein mono-salt?

2. What is the relation between the two series of compounds previously called casein mono-salts and casein di-salts?

3. What is the relation of casein and its derivatives to para-casein and its derivatives?

It may be well to state here, once for all, that our work deals exclusively with the casein of cows' milk and products derived from it.
THE RELATION OF MILK-CASEIN TO THE CASEIN COMPOUND SOLUBLE IN DILUTE SALT SOLUTIONS.

A study of the relation of milk-casein to the salt-soluble substance, which we have previously called a casein mono-salt, naturally resolves itself into a study of the two following questions:—(1) What is milk-casein, especially in relation to inorganic compounds? and (2) What is the salt-soluble compound formed from milk-casein?

THE RELATION OF INORGANIC COMPOUNDS TO MILK-CASEIN.

Does casein, as it is found in cows' milk, exist as a free proteid or is it in some form of combination with an inorganic compound?

Hammarsten, who must be regarded as the most successful pioneer worker in this field, has not clearly and specifically stated what he considers milk-casein to be. He does not appear to make any chemical distinction between ash-free casein, as prepared from cows' milk by his well-known method, and casein as it is found in milk. He appears to favor the idea that there is in milk-casein some kind of a physical relation between the proteid and calcium phosphate, casein acting as a solvent for calcium phosphate. Eugling held that casein enters into combination with tricalcium phosphate, and the same view was held by Schaffer. Söldner showed that casein prepared from cows' milk can form two calcium salts, (1) a basic calcium casein, containing about 2.36 per ct. of CaO, neutral to phenolphthalein, but alkaline to litmus, and (2) a neutral calcium casein, containing about 1.55 per ct. of CaO, slightly acid to phenolphthalein and neutral or slightly alkaline to litmus. Söldner believed that this neutral calcium casein is probably the form existing in milk, owing to its reaction and to its coagulability by rennet in the presence of soluble calcium salts. He also held that the calcium phosphate, usually found in casein precipitates, exists in a condition of

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suspended particles finely divided, and not in chemical combination with casein. Courant\(^7\) confirmed the work of Söldner, calling the basic compound tri-calcium casein, and the neutral compound di-calcium casein. He also reported on rather intangible evidence a mono-calcium casein, the existence of which has not been confirmed and appears doubtful. He regards milk-casein as made up partly of di-, and partly of mono-, calcium casein. Courant's work was entirely volumetric and no attempt was made to isolate the individual compounds. Lehman and Hempel\(^8\) regard milk-casein as a double compound formed by the combination of calcium casein and calcium phosphate.

The work done by us confirms that of Söldner. While he carried on his work mostly by volumetric methods, we have succeeded in isolating the two calcium casein compounds described by him. The details of our work follow.

*Preparation of casein.*—We prepared casein from cows' milk by Hammarsten's method, repeating the precipitation and solution four times. The precipitate was finally dissolved in 20 liters of very dilute ammonia water and precipitated by dilute acetic acid. The precipitate was ground in a mortar several times with water, then with alcohol, after which it was treated with ether and dried at 100\(^\circ\) C. Thus prepared, the casein was practically ash-free, containing only .042 per ct. of ash. This preparation was still slightly soluble in warm salt solution and in hot 50 per ct. alcohol.

*Behavior of casein preparation toward calcium carbonate.*—We rubbed in a mortar .5 gram of the casein preparation described above with finely divided calcium carbonate, suspended in water. The mixture was filtered several times through paper, giving a slightly opalescent filtrate. This solution was precipitated by 95 per ct. alcohol, the precipitate was washed several times with alcohol, then treated with ether and dried at 100\(^\circ\) C. This preparation contained 2.46 per ct. of CaO, agreeing very closely with Söldner's work in which he showed by an indirect method that a casein preparation treated with calcium carbonate combined with 2.39 per ct. of CaO. A part of this calcium casein preparation was dissolved in dilute ammonia, reprecipitated by

\(^7\)Pflüger's Arch., 50 : 109 (1891).
\(^8\)Pflüger's Arch., 56 : 558 (1894).
dilute acetic acid and the precipitate, after thorough washing and filtering, was again obtained practically ash-free.

_Solubility of casein preparation in lime-water._—We dissolved 3 grams of the casein preparation in lime-water and obtained an opalescent solution.

_Preparation of basic calcium casein._—The lime-water solution of casein was made neutral to phenolphthalein by $\frac{1}{10}$ hydrochloric acid and became somewhat more milky. This solution, neutral to phenolphthalein, was treated with 95 per ct. alcohol, and the resulting precipitate, which readily separated, was washed several times with alcohol, finally with ether and dried at 100$^\circ$ C. This precipitate contained calcium chloride, either as the result of occlusion or possibly of a loose combination with the proteid. In several preparations made, calcium chloride was always present in amounts varying from .75 to 1.2 per ct. Chlorine and calcium oxide determinations were made with the following results: 1.24 grams of material yielded .0331 gram of CaO. There was present .0063 gram of Cl, equivalent to .0097 gram of CaCl$_2$ or .0047 gram of CaO. This would leave .0284 gram of CaO combined with casein, or 2.29 per ct. This preparation was therefore the basic calcium casein of Söldner. It was insoluble in 5 per ct. salt solution and in hot 50 per ct. alcohol.

_Preparation of neutral calcium casein._—When a solution of casein in lime-water is made neutral to phenolphthalein, the basic calcium casein is formed, containing 2.29 to 2.46 per ct. of CaO. When the addition of acid is carried farther, a point is reached where the solution becomes neutral to litmus and much more milky in appearance than the solution neutral to phenolphthalein. A solution of casein in lime-water was made neutral to litmus by the addition of $\frac{1}{10}$ hydrochloric acid; to this solution alcohol was added, throwing down a white precipitate. The precipitate was washed with alcohol and ether and dried at 100$^\circ$ C. This preparation contained 1.13 per ct. of CaCl$_2$. On analysis we found that 1.27 grams gave .0257 gram of CaO and .0093 gram of Cl (equivalent to .0072 gram of CaO$_2$) leaving .0185 gram of CaO or 1.46 per ct. as combined with the proteid. Söldner recognized this combination and attributed to it a content of 1.55 per ct. of CaO. Another preparation contained
0.86 per ct. of CaCl₂ and 1.48 per ct. of CaO combined with casein.

Behavior of calcium casein solution toward rennet enzym and calcium chloride.—A solution of basic calcium casein, the compound neutral to phenolphthalein, is not coagulated by rennet enzym either in the presence or absence of soluble calcium salts, as calcium chloride. A solution of neutral calcium casein, the compound neutral to litmus, is coagulated by rennet, in the presence of soluble calcium salts. When the soluble calcium salts are removed by ammonium oxalate, no coagulation occurs. These statements are in agreement with the observations of others.

Casein present in milk as neutral calcium casein.—Our results appear to confirm in every respect the work of Söldner in regard to the relation of inorganic compounds to milk-casein. We have had the advantage of isolating the two compounds, which with him were more largely matters of inference. Of these two calcium casein compounds, the one containing about 1.55 per ct. of CaO and neutral to litmus and coagulable by rennet enzym in the presence of soluble calcium salts, appears to meet all the requirements of milk-casein. So far as our present knowledge goes, we seem to be justified in regarding milk-casein as a compound of the base calcium oxide (1.55 per ct.) and the ash-free proteid casein.

The behavior of calcium casein compounds toward soluble calcium salts.—Basic calcium casein, prepared by dissolving free casein in lime-water and making neutral to phenolphthalein by acid, is coagulated by soluble calcium salts on warming to 35° to 45° C. The coagulum is insoluble in warm 5 per ct. salt solution. If any soluble calcium salts present are removed by treatment with ammonium oxalate, the basic calcium casein is not coagulated on warming. Neutral calcium casein, prepared by dissolving free casein in lime-water and making neutral to litmus, is coagulated on warming by soluble calcium salts. The coagulum is insoluble in dilute salt solution. The neutral calcium casein existing in milk is coagulated on warming by a few drops of a solution of calcium chloride. If we add to milk an amount of acid insufficient to cause coagulation or to remove from its combination the calcium of the calcium casein, we can produce some coagulation
on warming. This is probably due to the conversion of insoluble into soluble calcium salts, and the same result follows as when we add a soluble calcium salt directly to the milk.

The coagulation of calcium casein compounds by a soluble calcium salt may be due to purely physical change in the calcium casein compounds or there may be a loose chemical combination between the soluble calcium salt and the calcium casein compounds, the resulting compound being insoluble in the neutral or slightly acid medium.

**THE RELATION OF THE SALT-SOLUBLE COMPOUND OF CASEIN TO MILK-CASEIN.**

The preparations of calcium casein, while slightly soluble in hot 50 per ct. alcohol, were insoluble in warm 5 per ct. salt solution. None of these preparations showed in any marked degree the properties of the compounds that we have called casein mono salts. We have already called attention to the fact that, in making an ash-free preparation of casein from cows' milk we found it to be readily soluble in both warm dilute salt solution and in 50 per ct. hot alcohol. This suggested that there might be a close relation between these compounds and we therefore made several preparations, the details of which we now give.

*Preparation 1, by use of hydrochloric acid.*—We diluted 2 liters of skimmed milk with 5 volumes of distilled water and warmed the mixture to 45° C. Dilute hydrochloric acid was added, accompanied by vigorous stirring to keep the precipitate in a finely divided condition. After the precipitate had settled, the supernatant liquid was poured off, the precipitate was triturred in a mortar with water and finally allowed to drain on a filter paper. It was then removed from the filter paper, mixed with water at 45 to 50 C. triturated again and filtered again. This was repeated four or five times a day and continued until the filtrate gave no test for chlorides. This required four days. When the precipitate had finally been drained, it was washed with 95 per ct. alcohol. Some of the proteid went into solution, owing to dilution of the alcohol by the water still present in the precipitate; and this partial solution stopped only after a large volume of alcohol had been used. The precipitate was finally
washed with ether and dried at 100° C. The product thus prepared was a very friable white powder. It was still somewhat soluble in hot 50 per ct. alcohol and warm 5 per ct. salt solution. These solubilities are much more marked before treatment with strong alcohol and ether. The product was practically ash-free, 5.28 grams yielding an ash of .02 per ct. The preparation was quite free from chlorides, this being shown in the following manner:—We dissolved .5 gram in hot concentrated nitric acid, to which was added a crystal of silver nitrate. The whole was boiled until complete solution of the proteid had occurred. No precipitate of silver chloride was formed.

Preparation 2, by Hammarsten's method.—We used 2 liters of skim-milk in making this preparation and followed in exact detail the method described by Hammarsten. Before treatment with alcohol, this preparation was readily soluble in warm dilute salt solution and in 50 per ct. hot alcohol. It contained 1.06 per ct. of ash. It was shown to be free from acetates in the following manner:—We dissolved .5 gram in dilute sodium hydroxide, then precipitated the proteid by hydrochloric acid and filtered. The filtrate was made alkaline and evaporated to dryness. The residue gave no evidence of ethyl acetate when heated with alcohol and sulphuric acid.

Preparation 3, by use of hydrochloric acid.—We used 2 liters of skim-milk, diluting it with 15 liters of warm distilled water, and precipitating the casein by hydrochloric acid. The separated proteid was allowed to drain on filter paper and then washed with water at 45° C. It was then mixed with water and triturated and washed several times. The precipitate soon showed marked solubility in warm dilute salt solution and in hot 50 per ct. alcohol. The washing was continued until all chlorides were removed. The precipitate was finally washed with alcohol and ether and dried at 100° C. It was free from chlorides. Its solubility was now slight in alcohol or salt solution. The ash content was .81 per ct. We have noticed that the degree of fineness of the proteid after precipitation largely influences the ash content.

Preparation 4, by use of sulphuric acid.—The same method was

followed as that described in preparation 3, except that we used sulphuric acid as the precipitant instead of hydrochloric acid. Triturating this preparation under different portions of water four or five times a day, we had to continue the operation 19 days before the filtrate failed to show the presence of sulphuric acid. A little chloroform was added from time to time to prevent bacterial action. The proteid was found to be entirely free from sulphuric acid. The extreme difficulty met in this case in removing the sulphuric acid was due to the formation of calcium sulphate in the first precipitation and to its slow solubility in water. This preparation had an ash content of .56 per ct. During the process of removing the sulphuric acid, the precipitate was readily soluble in hot 50 per ct. alcohol and in warm five per ct. solution of sodium chloride. After treatment with alcohol, these solubilities were almost entirely lost.

Preparation 5, made from lime-water solution of casein.—If we dissolve an ash-free preparation of casein in lime-water and add to this solution $\frac{n}{10}$ hydrochloric acid until blue litmus is just turned red, no visible separation of proteid occurs. If we continue to add acid, the proteid separates; and complete precipitation of the casein occurs, when all the calcium in the original lime-water solution has been neutralized. This can be shown quantitatively in the following manner: Of the lime-water employed, 50 cc. required for neutralization 19.2 cc. of $\frac{n}{10}$ hydrochloric acid, using phenolphthalein as an indicator. We dissolved .5 gram of ash-free casein in 50 cc. of this lime-water. On titration, the neutral point with phenolphthalein was reached when we had used 15.2 cc. of $\frac{n}{10}$ hydrochloric acid. This means that calcium oxide, equivalent to 4 cc. of $\frac{n}{10}$ hydrochloric acid, was no longer present as calcium hydroxide, that is, .0112 gram of calcium oxide. This amount had combined with the .5 gram of proteid, forming 2.24 per ct. of the calcium proteid compound, and this proportion indicates the basic calcium casein.

When we had added 16.4 cc. of $\frac{n}{10}$ hydrochloric acid, the solution was just blue to litmus. This indicates that calcium oxide, equivalent to 2.8 cc. of $\frac{n}{10}$ hydrochloric acid, or .00784 gram of calcium oxide, was no longer present as calcium hydroxide. This amount had combined with the .5 gram of proteid, forming
1.57 per ct. of the calcium proteid combination, and this proportion indicates the neutral calcium casein.

When we had added 16.6 cc. of \( \frac{7}{10} \) hydrochloric acid to the lime-water solution of casein, litmus was turned red, but no precipitation occurred yet. After 18.5 cc. of \( \frac{7}{10} \) hydrochloric acid had been added, a precipitate began to form, and the precipitation of the dissolved casein was complete when we had added 19.2 cc. of \( \frac{7}{10} \) hydrochloric acid, or just enough to combine with all the calcium originally present in the solution used. This work was repeated with closely agreeing results. *This precipitate is therefore base-free casein.* A preparation made in this manner and thoroughly washed showed that all the calcium present was there as calcium chloride, either occluded in the precipitate or possibly in a loose form of combination with the proteid,—in any case there was no calcium base in combination with the casein. The precipitate contained in one gram, .00343 gram of Ca and .0064 gram of Cl, which amounts agree well for the presence of calcium chloride. This body, prepared by neutralizing a solution of calcium casein, is insoluble in water but readily soluble in warm dilute sodium chloride solution and in hot 50 per ct. alcohol.

In another experiment, 25 cc. of lime-water required 10.6 cc. of \( \frac{7}{10} \) hydrochloric acid to neutralize it. We dissolved in this amount of lime-water .5 gram of ash-free casein, prepared according to Hammarsten. To precipitate this amount of casein completely required 10.6 cc of \( \frac{7}{10} \) hydrochloric acid. The precipitated substance was soluble in warm dilute salt solution and in hot dilute alcohol. To this substance we added .5 cc of \( \frac{7}{10} \) hydrochloric acid and obtained a substance insoluble in warm dilute salt solution and only slightly soluble in hot dilute alcohol.

When the product, made by neutralizing the lime-water solutions of ash-free casein with hydrochloric acid, was dissolved in hot 50 per ct. alcohol, the dissolved proteid separated from the alcoholic solution on cooling, forming on the bottom of the beaker a gummy, sticky mass, which could easily be gathered on the end of a glass rod. When the body, freshly precipitated from its solution in lime-water, is warmed on the water-bath, it can similarly be easily gathered on a glass rod in an adherent
gummy mass. When warm, it is plastic and can be drawn out in fine, long, silky threads.

Identity of base-free casein and salt-soluble body.—In the presentation of facts preceding, we have seen that when an ash-free, that is, base-free proteid, or one practically so, is prepared by precipitating milk-casein (calcium casein), with an acid, the acid precipitant being completely removed from the proteid, or by treating a lime-water solution of a base-free casein with an acid to the point of acidity with litmus, we obtain a body which is soluble in warm 5 per cent. salt solution and in hot 50 per cent. alcohol. This body, when freshly prepared and warmed, is very plastic and is capable of being drawn into very long, fine, silky threads. It behaves in all respects like the compound which we were led to regard at first as a compound formed by combination of casein and an acid and which we regarded as a casein mono-salt of the acid precipitant. As a result of this work, we now believe that the compound formed by treating milk with an amount of acid just sufficient to combine with the calcium of the calcium casein, in addition to certain inorganic salts of the milk, is not a casein mono-salt of the acid but is base-free casein, or milk-casein (calcium casein) from which the calcium has been removed by combination with acid.

THE RELATION BETWEEN THE TWO SERIES OF COMPOUNDS PREVIOUSLY CALLED CASEIN MONO-SALTS AND CASEIN DI-SALTS.

We have already seen that when calcium casein (milk-casein) is treated with dilute acid, the base is removed from its combination with the proteid and the base-free proteid is formed, a compound corresponding in its properties to those of the compounds we formerly called casein mono-salts of acids. When to this base-free proteid we add a dilute acid, another body appears to be formed, which is insoluble in dilute salt solution and in hot 50 per cent. alcohol and which differs also in a marked loss of the plastic properties exhibited by the base-free proteid. This is the familiar substance ultimately formed when milk is coagulated by ordinary souring or by direct addition of acids in sufficient quantity. The formation of this substance has usually been explained in
two different ways: (1st) It has been quite generally held that the acid unites with the inorganic portion (calcium) of the milk-casein, thus destroying the combination, and that the free proteid then appears as a solid. (2d) It has been held by some that the acid actually combines with the proteid, forming a casein salt of the acid used. According to the first explanation, only one substance is formed when milk-casein is treated with an acid, forming a precipitate, and this one substance is the base-free casein. According to the second explanation, two substances are formed, one after the other, by treating milk-casein with an acid, viz.: (1st) the base-free casein and (2d) a compound formed by the combination of the base-free casein with the acid.

Now, the existence of one body with two different sets of properties, or of two different bodies, differing in their properties, must be acknowledged, when we treat milk-casein with an acid in proper proportions. When, by treating calcium casein with a certain amount of acid, we obtain the base-free proteid and then, by treating this with an additional amount of acid, we obtain a body differing in properties from the base-free proteid, the difference must either be due to chemical combination of the proteid and acid or else be the result of a purely physical change caused by the acid. Contrary to the view generally held, we have believed that there is actual combination between the proteid and acid, forming a casein salt of the acid, and this we formerly called a casein di-salt of the acid used. Since we have shown that our supposed casein mono-salts of acids are simply the base-free casein, it would appear that there is only one series of casein compounds formed by combination with acids, existing as a precipitate.

Hammarsten held that there could be no combination between the casein and acid, because by trituration with water in a mortar he was able to remove the acid completely from the proteid. We have abundantly confirmed his statement that the acid can be removed from the proteid by trituration, but in view of recent developments in chemistry, this argument has no weight, since we can do the same thing in the case of some well-known and well-established inorganic salts. For example, the sulphuric
acid radical of mercuric sulphate can be completely removed by trituration with water.

EXPERIMENTS SHOWING COMBINATION OF CASEIN AND ACIDS.

In order to show that there is actual combination between base-free casein and acids, we used the following method: We suspended in 50 cc. of distilled water .5 gram of finely-ground base-free casein and allowed the mixture to stand one hour, with occasional shaking. At the end of this time, we added hydrochloric acid of known strength in definite quantities, after which the volume was made to 100 cc. by water and filtered. The residue was not washed. Aliquot parts of the filtrate were titrated with \( \frac{n}{10} \) sodium hydroxide, using phenolphthalein as indicator. The difference between the amount of acid added and that found in the filtrate represents the amount of acid combining with the casein, plus the amount adhering mechanically to the casein. It was necessary to use dilute solutions of acid in order to avoid dissolving the proteid by an excess of acid. The materials used in this work were the four preparations of base-free casein which have been already described on pp. 11-13.

Amount of acid used kept constant in relation to proteid.—In the experiments, the results of which are given in Table I, we used different solutions of hydrochloric acid of varying dilution, but varied the quantity of acid used so that the same absolute amount of acid was used in each case, which was equal to 20 cc. of \( \frac{n}{10} \) hydrochloric acid for one gram of base-free casein. The work was carried on at room temperature, 17° to 18° C.
### Table I.—Amount of Hydrochloric Acid Neutralized by One Gram of Base-Free Casein with Constant Amount of Acid.

<table>
<thead>
<tr>
<th>No. of cc. of acid used</th>
<th>Strength of acid used</th>
<th>Preparation 1</th>
<th>Preparation 2</th>
<th>Preparation 3</th>
<th>Preparation 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n-100 HCl in filtrate</td>
<td>n-100 HCl in protein</td>
<td>n-100 HCl in filtrate</td>
<td>n-100 HCl in protein</td>
</tr>
<tr>
<td>40</td>
<td>n-200</td>
<td>cc</td>
<td>cc</td>
<td>cc</td>
<td>cc</td>
</tr>
<tr>
<td></td>
<td>n-100</td>
<td>13.6</td>
<td>6.4</td>
<td>13.6</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>13.6</td>
<td>6.4</td>
<td>13.6</td>
<td>6.4</td>
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<tr>
<td></td>
<td>12</td>
<td>14.0</td>
<td>6.0</td>
<td>14.0</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>14.0</td>
<td>6.0</td>
<td>14.0</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>13.6</td>
<td>6.4</td>
<td>13.6</td>
<td>6.4</td>
</tr>
<tr>
<td>Av'ge</td>
<td></td>
<td>13.7</td>
<td>6.3</td>
<td>13.8</td>
<td>6.2</td>
</tr>
</tbody>
</table>

**Amount of acid used varying in relation to protein.**—In another set of experiments, we used varying quantities of acid for one gram of protein. Otherwise the experiment was carried out like those preceding. The results are given in Table II.

### Table II.—Amount of Hydrochloric Acid Neutralized by One Gram of Base-Free Casein, the Amounts of Acid Varying.

<table>
<thead>
<tr>
<th>No. of cc. of acid used</th>
<th>Strength of acid used</th>
<th>Amount of acid used</th>
<th>Preparation 1</th>
<th>Preparation 2</th>
<th>Preparation 3</th>
<th>Preparation 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cc</td>
<td>cc</td>
<td>cc</td>
<td>cc</td>
<td>cc</td>
</tr>
<tr>
<td>30</td>
<td>n-200</td>
<td>8.8</td>
<td>6.2</td>
<td>9.2</td>
<td>5.8</td>
<td>8.6</td>
</tr>
<tr>
<td>20</td>
<td>n-100</td>
<td>13.6</td>
<td>6.4</td>
<td>13.2</td>
<td>6.8</td>
<td>13.4</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>19.2</td>
<td>5.8</td>
<td>19.2</td>
<td>5.8</td>
<td>19.0</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>16.6</td>
<td>5.8</td>
<td>16.6</td>
<td>5.8</td>
<td>16.8</td>
</tr>
<tr>
<td>Average.................</td>
<td></td>
<td>6.0</td>
<td>—</td>
<td>6.1</td>
<td>—</td>
<td>6.2</td>
</tr>
</tbody>
</table>

**Amount of acid and amount of dilution varied in relation to protein.**—The amount of water in which the solid base-free casein was suspended was relatively considerable in the preceding experiments. We wished to see what difference if any, there might be when we treated the casein directly with acids of varying strength and without farther dilution. We sus-
pended one gram of the base-free casein in each of the following amounts of solutions of hydrochloric acid: 50 cc. of \(\frac{8}{10}\), 40 cc. of \(\frac{6}{10}\), 30 cc. of \(\frac{5}{10}\), and 20 cc. of \(\frac{4}{10}\). The mixtures were allowed to stand one hour with occasional agitation and were then filtered and aliquot portions of the filtrate titrated as in the preceding experiments. The results are given in the following table:

**Table III.—Amount of Hydrochloric Acid Neutralized by One Gram of Base-Free Casein, Amounts and Dilution of Acid Varying.**

<table>
<thead>
<tr>
<th>No. of cc. of acid used.</th>
<th>Strength of acid used.</th>
<th>Amount of acid used equal to n-100 HCl</th>
<th>Acid equal to n-100 HCl in filtrate</th>
<th>Acid equal to n-100 HCl in proteid</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>n-200</td>
<td>25</td>
<td>18.4</td>
<td>6.6</td>
</tr>
<tr>
<td>40</td>
<td>n-100</td>
<td>40</td>
<td>33.6</td>
<td>6.4</td>
</tr>
<tr>
<td>30</td>
<td>n- 80</td>
<td>37.5</td>
<td>30.0</td>
<td>7.5</td>
</tr>
<tr>
<td>20</td>
<td>n- 60</td>
<td>33.3</td>
<td>25.6</td>
<td>7.7</td>
</tr>
</tbody>
</table>

The results in Table III indicate that when we treat the base-free casein with a more concentrated acid solution, we have more acid left in the proteid. This is as we should expect under the conditions of experiment. The acid that is not recovered in the filtrate must either combine with the proteid or adhere to it mechanically. We made no attempt to wash the adherent or occluded acid free from the proteid, since any thorough washing would remove some of the combined acid. Therefore, the more concentrated the acid used in treating the proteid, the more acid, as measured by titration, adhered to the proteid in addition to the fixed amount of acid that combined with the proteid, as compared with treatment with an equal volume of more dilute acid.

It is not easily conceivable that such an amount of acid as is represented by about 6 cc. of \(\frac{8}{10}\) hydrochloric acid should be held only mechanically by one gram of casein, when we used solutions so dilute as those with which we worked. Bearing on this point the following additional experiments were made:

(1) We suspended a weighed amount of base-free casein in 50 cc. of water, let stand 15 minutes, added \(\frac{8}{10}\) hydrochloric acid at the rate of 6 cc. for one gram of casein and made the volume to
100 cc. with water. After filtration, the filtrate was titrated and the result showed that 5.2 cc. of $\frac{9}{10}$ hydrochloric acid had been held by one gram of casein.

(2) The experiment was repeated, allowing the mixture to stand one hour after addition of acid. The result was the same as before.

(3) The experiment was repeated, except that we used only 4 cc. of $\frac{9}{10}$ hydrochloric acid for one gram of casein. The filtrate on titration showed no acid, indicating that the entire 4 cc. of $\frac{9}{10}$ hydrochloric acid had been held by the casein.

These results indicate to us that one gram of base-free casein combines with about .5 cc. of $\frac{9}{10}$ hydrochloric acid to form a compound of casein and hydrochloric acid, and that the disappearance of acid is due to chemical union with proteid and not merely to mechanical mixture or adhesion.

The question has probably come to mind before this as to why free casein was not sooner isolated in the normal souring of milk or in the treatment of milk by direct addition of acid. It can now readily be seen why this was so. Since it requires so little acid to change the free casein into its acid combination, the point is quickly passed when we have any considerable proportion of free casein in the milk. In the normal souring of milk we were able to catch the change at a point when 65 per ct. of the casein of the milk was in the form of free casein, as shown in Bulletin No. 245, p. 12. When we use an acid to precipitate casein from milk, an excess is added, so that we get, not the free casein, but the compound formed by its combination with acid.

**Summary of action of acids on milk-casein. (calcium casein).**—When the calcium casein of cows' milk is treated with an acid, the first reaction that takes place is a union between the acid and the calcium combined with the casein, resulting in the formation of base-free casein, a compound insoluble in water but soluble in warm 5 per ct. solution of sodium chloride and in hot 50 per ct. alcohol; a compound which also possesses the property, when warmed, of being very plastic and capable of being drawn out into long, fine, silky threads. This base-free casein is identical with the compound which we formerly regarded as being a casein mono-salt of the acid used as precipitant.
When one gram of this base-free casein is treated with an amount of acid equivalent to about .5 cc. in the case of \( \frac{n}{10} \) hydrochloric acid, the properties of the casein are changed, so that it is no longer soluble in 5 per ct. salt solution and only slightly soluble in hot 50 per ct. alcohol; and, in addition, it has lost entirely its plastic properties and the power of being drawn out into fine threads when warm. This substance we regard as resulting from the combination of acid with the base-free casein, forming a casein salt of the acid used. Our reasons for believing that there is actual chemical combination between the casein and acid are the following:

(1st). When a given amount of base-free casein is treated with an acid, a quite definite and constant amount of acid appears to be neutralized. In the case of \( \frac{n}{10} \) hydrochloric acid, one gram of base-free casein appears to combine with about .5 cc. of acid.

(2d). The phenomenon of combination between proteids and acids appears to be very general, as shown by Cohnheim and others.\(^{10}\)

(3d). When base-free casein is treated with an acid, it undergoes a marked change in its properties.

(4th). While the combination is comparatively weak, it appears to be as strong as in the case of some quite stable inorganic salts. The fact that the acid can be completely removed by long-continued trituration with water does not constitute an argument against combination, since some well-known inorganic salts behave the same way under similar treatment.

(5th). By treating base-free casein with a given amount of acid, we form the compound insoluble in warm dilute salt solution and hot alcohol. By removing the acid from this latter compound, we obtain again the base-free casein with a restoration of its recognized properties of solubility and plasticity. Each of these compounds can be converted into the other by addition or removal of acid.

\(^{10}\) Chemie der Eiweisskörper, pp. 106, 114, 2d Ed. 1904.
THE RELATION OF CASEIN AND ITS COMPOUNDS TO PARACASEIN AND ITS COMPOUNDS.

The coagulum formed by the calcium casein of cows' milk when treated with rennet enzym was called by Hammarsten" "Käse." Schulze and Röse" suggested the name "paracasein" for this product and their suggestion has been very generally accepted. There is still, however, much confusion as to the exact application of this term, as we shall point out later.

According to Hammarsten and others, the action of rennet enzym on calcium casein of milk takes place in two distinct stages, as follows: (1) The rennet enzym converts the milk-casein into paracasein, but there is no coagulation or change visible to the eye, the paracasein remaining in the same condition apparently as the milk-casein. In the absence of soluble calcium salts, the paracasein remains in this uncoagulated form in the case of normal milk. (2) In the second stage, coagulation or separation of the curd takes place in the presence of soluble calcium salts. The conversion of milk-casein into paracasein in the first stage is due to the rennet enzym alone, while the coagulation of the paracasein in the second stage is due to soluble calcium salts alone in the case of normal milk. The term paracasein is commonly applied to both the coagulated and uncoagulated forms.

Just what chemical change in the proteid, if any, takes place in the conversion of calcium casein into paracasein by the rennet enzym, has not been clearly demonstrated. According to Hammarsten's original theory the calcium casein of the milk undergoes a hydrolytic splitting by the action of rennet enzym into two compounds: (1) A body difficulty soluble, forming the chief product and closely resembling casein in composition, paracasein; and (2) an easily soluble, albumose-like body, called by him whey-proteid, produced in very small amount. The paracasein further has not the property of holding calcium phosphate in solution to the same extent that casein has. Ham-

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13 Maly Jahresber. d. Tierchem., 2: 118 (1872) and 7: 158, (1877).
marsten\textsuperscript{15} later modified these views, coming to the conclusion that the action of rennet enzyme rearranges the casein molecule only in a physical way. Du Claux\textsuperscript{16} regarded casein as existing in milk in three different forms in equilibrium with one another; this equilibrium could be easily disturbed by the action of acids or ferments. Coagulation, according to his theory, was a purely physical, mechanical change. A similar conclusion is held by Fuld\textsuperscript{17}, milk coagulation being regarded as a special case of the alternation between suspension and precipitation of a colloidal substance. Eulgling\textsuperscript{18} concluded from work done by him that acids or rennet, when added to milk, render the insoluble calcium salts soluble and more readily available for the coagulation of the proteid. De Vries and Boekhout\textsuperscript{19} believe that free acid exerts upon the coagulation of paracasein a direct influence in some way on its own account, independent of the formation of soluble calcium salts. Loevenhart\textsuperscript{20} has stated that there is no chemical difference between casein and paracasein, and that observed differences in properties are due to physical changes, casein and paracasein being physical modifications of one and the same body. According to his belief, paracasein exists in larger molecular aggregations than does casein.

We thus see that, while there is a wide range of opinion in regard to the relation of casein and paracasein, there is a prevailing tendency to regard these proteids, milk-casein and paracasein, as being essentially alike in composition.

We have already referred to the circumstances which led us to regard as a mono-lactate of paracasein a body which we were able to separate from cheese by means of a warm 5 per ct. salt solution. We have indicated how we came to regard the substance formed from this body by treatment with lactic acid as paracasein dilactate. We have in the preceding pages shown how we now regard the corresponding casein compounds. It is

\textsuperscript{15} Zeit. f. Physiol. Chem., 28: 114 (1899).
\textsuperscript{16} Compt. Rend., 98: 373.
\textsuperscript{17} Beiträge z. Chem. Physiol. und Pathol., 2: 169 (1902).
\textsuperscript{18} Landwirth. Versuchs-Stat., 31: 392 (1885).
\textsuperscript{20} Zeit. Physiol. Chem., 41: 177 (1904).
now our purpose to consider in a similar manner paracasein and some of its compounds, and we shall present our discussion in the following order:

1. The relation of inorganic compounds to paracasein.
2. The relation of the salt-soluble compound of paracasein to paracasein.
3. The relation between the two series of compounds previously called paracasein mono-salts and paracasein di-salts.
4. The relation of casein and its compounds to paracasein and its compounds.

**The relations of inorganic compounds to paracasein.**

In order to study paracasein in its relations to calcium and to acids, it was essential to obtain a paracasein that should be practically base-free. Preparations, made by coagulating cows' milk with rennet and washing the coagulum, are high in ash content, containing often 4 or 5 per ct.; moreover, such preparations still contain the calcium originally combined in the calcium casein of the milk and are not satisfactory for such work as we desired to do. We therefore made a preparation of paracasein as follows:

*Preparation of paracasein.*—To one liter of skim-milk, we added 1.25 grams of ammonium oxalate; the milk, after standing a short time, was filtered through paper. To 500 cc. of this filtrate we added .05 cc. of Hansen's rennet extract and kept the whole at a temperature of 37° C. for two hours. A small amount of thymol was added. To show that the rennet enzym had acted upon the calcium casein in the milk, we took 10 cc. of the treated milk, cooled to room temperature, and added two drops of a 10 per ct. solution of calcium chloride. In a few minutes the milk had formed a firm coagulum, which indicated that the rennet enzym had functioned and produced paracasein. The 500 cc. of treated milk we now diluted with 16 liters of warm, distilled water and added dilute hydrochloric acid until the proteid had separated. This precipitation was accompanied by vigorous stirring to keep the proteid as finely divided as possible, thus facilitating the removal of inorganic salts. After the precipitate had settled, the supernatant liquid was removed, the precipitate was filtered and then triturated with warm water and filtered,
the trituration and filtering being repeated until all trace of chlorides was removed, which required four days. The precipitate after removal of acid was soluble in warm 5 per ct. salt solution and in hot 50 per ct. alcohol. Finally, the preparation was washed with strong alcohol and ether and dried at 100° C. This treatment greatly lessened its solubility in dilute salt solution and alcohol. It was nearly ash-free, containing 0.11 per ct. and gave no test for chlorides when boiled with nitric acid and silver nitrate.

Solubility of paracasein preparation in lime-water.—The paracasein, prepared in the manner described, was soluble in lime-water, forming a solution having a dull, opalescent appearance.

Preparation of basic calcium paracasein.—We dissolved about 2 grams of our paracasein preparation in lime-water and made the solution neutral to phenolphthalein by addition of 1/8th hydrochloric acid. The solution became more milky in appearance but no precipitation was observable. This solution was now precipitated by alcohol, filtered, washed several times with alcohol, finally with ether and dried at 100° C. The preparation was insoluble in dilute salt solution and practically so in hot dilute alcohol. Determinations of calcium and chlorine gave the following results: One gram contained a total of .0283 gram of CaO, and .0063 gram of Cl (equivalent to .0047 gram of CaO combined as calcium chloride). Deducting the calcium oxide equivalent, present as chloride, from the total, we have .0236 gram, which represents the amount of calcium oxide combined with the proteid, or 2.36 per ct. This preparation therefore corresponds very closely in its calcium content, with the basic calcium casein. As in the case of the calcium casein preparations, all the calcium paracasein preparations contained calcium chloride.

Preparation of neutral calcium paracasein.—When we treat a lime-water solution of paracasein with an amount of acid sufficient to turn blue litmus red, or just a little short of this, the proteid separates as a precipitate and this occurs before all the calcium of the original lime-water solution has been neutralized. This product is insoluble in dilute salt solution and nearly insoluble in hot dilute alcohol. The product was thrown on a filter...
and washed several times with water. Filtration was rather slow. The precipitate was then washed with alcohol and ether and dried at 100° C. Determinations of calcium and chlorine gave the following results: One gram contained .0391 gram of ash, a total lime content of .0194 gram of CaO, and .0064 gram of Cl (equivalent to .0048 gram of CaO combined as calcium chloride). Deducing the CaO equivalent, present as chloride, from the total CaO, we have .0146 gram, which represents the amount of calcium oxide combined with the proteid or 1.46 per cent. This preparation therefore corresponds closely to the neutral calcium casein.

We have seen above that the neutral calcium paracasein, formed by making a lime-water solution of paracasein neutral to litmus by acid, separates as a precipitate, while neutral calcium casein does not separate thus. At the point of neutrality with litmus, the body formed, that is, neutral calcium paracasein is precipitated. In this respect and in this respect only does there appear to be a marked difference between the properties of casein and paracasein compounds. This is right in harmony with what takes place when rennet acts on milk in the presence of soluble calcium salts,—an insoluble calcium paracasein is formed. This shows that our preparation of paracasein had not reverted to casein.

Preparation of base-free paracasein.—In 50 cc. of lime-water, requiring 21.1 cc. of $\frac{n}{T_0}$ hydrochloric acid for neutralization, we dissolved .5 gram of our ash-free paracasein preparation. The solution became neutral to phenolphthalein after the addition of 16.9 cc. of $\frac{n}{T_0}$ hydrochloric acid. This leaves calcium oxide equivalent to 4.2 cc. of $\frac{n}{T_0}$ hydrochloric acid as combined with the proteid or .01176 gram of CaO, which is 2.35 per cent of the paracasein, and this is the basic calcium paracasein.

When 18 cc. of $\frac{n}{T_0}$ hydrochloric acid had been added, a precipitate began to separate, but the solution was still alkaline to litmus. When 18.5 cc. of the acid had been added, precipitation was complete. This leaves calcium oxide equivalent to 2.6 cc. of $\frac{n}{T_0}$ hydrochloric acid as combined with the proteid, or .00728 gram of CaO, which is 1.46 per cent of the paracasein, and this is the neutral calcium paracasein.

When 21.1 cc. of $\frac{n}{T_0}$ hydrochloric acid had been used, an
amount necessary to neutralize exactly the lime-water used for dissolving the paracasein, the proteid was still precipitated. It was now quite easily soluble in warm 5 per ct. salt solution and readily so in hot 50 per ct. alcohol. On warming, the precipitate separated on the bottom of the beaker and was easily gathered in a mass on a stirring rod. This mass was very plastic, showing a tendency to flow when kept warm; it also possessed the property of being drawn into long, fine, silky threads. It behaved in every respect like base-free casein.

In another experiment, we dissolved 1.5 grams of our paracasein preparation in lime-water and then exactly neutralized the solution with 1/10 hydrochloric acid. The precipitated proteid was washed with alcohol and ether and dried at 100° C. Determinations of calcium and chlorine showed their presence in the proportions found in calcium chloride, which was probably held mechanically in the proteid mass.

Behavior of lime-water solutions of paracasein compounds towards rennet and soluble calcium salts.—Basic calcium paracasein, prepared by dissolving base-free paracasein in lime-water and making neutral to phenolphthalein, is not coagulated by rennet in the presence or absence of soluble calcium salts. However, a solution of basic calcium paracasein may be coagulated on warming by soluble calcium salts alone without rennet.

Neutral calcium paracasein, prepared by dissolving base-free paracasein in lime-water and making neutral to litmus, is coagulated readily at room temperature or on warming by soluble calcium salts, with or without rennet, but not in the absence of soluble calcium salts.

Rennet changes calcium casein to calcium paracasein but does not coagulate the proteid. Soluble calcium salts coagulate neutral calcium paracasein and the action takes place through quite a range of temperature; while basic calcium paracasein is coagulated by soluble calcium salts only after warming. Here, as in the case of the corresponding calcium casein compounds, the coagulation may be the result of purely physical change or there may be a loose combination between the soluble calcium salt and the calcium paracasein compound.

Summary of results of work done on paracasein and its com-
pounds.—We have made and studied the following paracasein preparations: (1) Base-free paracasein, the free proteid, (2) basic calcium paracasein, containing about 2.40 per ct. of calcium oxide and (3) neutral calcium paracasein, containing about 1.50 per ct. of calcium oxide.

THE RELATION OF THE SALT-SOLUBLE COMPOUND OF PARACASEIN TO PARACASEIN.

Of the three paracasein preparations made and studied by us, we have found only one that was readily soluble in warm 5 per ct. salt solution and hot 50 per ct. alcohol, and this was the base-free paracasein. This body on warming also showed the peculiar plastic property and the power of being drawn into long, fine, silky threads, which are shown by the salt-soluble substance prepared by us from cheese-curd and cheese. This is the body which our former work led us to regard as a compound formed by combination of paracasein and lactic acid in the case of cheese and which we regarded as paracasein monolactate. As the result of our more recent work, we now believe that the compound formed by treating calcium paracasein (cheese curd) with an amount of acid just sufficient to combine with the calcium of the calcium paracasein, in addition to certain inorganic salts held mechanically in the cheese-curd, is not paracasein monolactate but base-free paracasein, or calcium paracasein from which the calcium has been removed by its combination with acid.

THE RELATION BETWEEN THE TWO SERIES OF COMPOUNDS PREVIOUSLY CALLED PARACASEIN MONO-SALTS AND PARACASEIN DI-SALTS.

When to base-free paracasein we add dilute acid, another body appears to be formed, which differs in properties from the base-free proteid in being insoluble in warm 5 per ct. salt solution and hot 50 per ct. alcohol, and also in possessing none of the peculiar plastic properties of the base-free paracasein. This substance is formed when milk is treated with rennet enzym and allowed to coagulate either by spontaneous souring or by the direct addition of dilute acids. We at first regarded this substance as a paracasein di-salt of an acid, but since we have shown that what we regarded as a paracasein mono-salt is
the base-free proteid, paracasein, we now regard this compound as resulting from the combination of acid with the base-free paracasein, forming a paracasein salt of the acid used, corresponding to the casein salts of acids, which have been already discussed.

In each of several experiments we suspended in distilled water portions of .5 gram of base-free paracasein and treated this with varying amounts of dilute acid of different strengths, agitating from time to time for an hour. The mixture was filtered and the filtrate titrated with $\text{T}_0^0$ hydrochloric acid. The amount of acid not recovered in the filtrate is regarded as representing approximately the quantity that had combined with the paracasein. This was found to be equivalent to about 0.5 cc. of $\text{T}_0^0$ hydrochloric acid, closely agreeing with the results obtained by treating base-free casein with acid.

**THE RELATION OF CASEIN AND ITS COMPOUNDS TO PARACASEIN AND ITS COMPOUNDS.**

We have made preparations of the following compounds, which have been described in the preceding pages:

*Casein*, the base-free proteid or uncombined proteid.

*Basic calcium casein*, containing the free proteid combined with about 2.40 per ct. of CaO.

*Neutral calcium casein*, containing the free proteid combined with about 1.50 per ct. of CaO.

*Casein salts of acids*, formed by combination of the free proteid with acids.

*Paracasein*, the base-free or uncombined proteid.

*Basic calcium paracasein*, free paracasein combined with about 2.40 per ct. of CaO.

*Neutral calcium paracasein*, free paracasein combined with about 1.50 per ct. of CaO.

*Paracasein salts of acids*, formed by combination of the free proteid with acids.

A comparison of the properties of casein and paracasein and their compounds strongly suggests that they are chemically alike, paracasein and its combinations being larger molecular aggregations than casein and its corresponding combinations, in accord-
ance with the suggestion of Loevenhart. The following statements serve to bring out the close resemblance more strikingly:

(1) The free proteids, casein and paracasein, possess the same solubilities in warm 5 per ct. salt solution and hot 50 per ct. alcohol. They possess the same peculiar plastic properties when warmed and show the same power of being drawn out in fine, silky threads.

(2) Casein and paracasein form compounds containing the same amounts of combined calcium oxide.

(3) Casein and paracasein, when treated with dilute acid in the proportion of one gram to about .5 cc. of \(\frac{n}{10}\) hydrochloric acid, are changed into bodies having the same properties, which differ strikingly from the properties of the free proteids.

(4) Neither basic calcium casein nor basic calcium paracasein is coagulated by rennet, either in the presence or absence of soluble calcium salts. Basic calcium casein and basic calcium paracasein are both coagulated when warmed to 35° to 45° C. in the presence of soluble calcium salts, but not in the absence of soluble calcium salts.

(5) Neutral calcium casein (present in milk) is coagulated by a few drops of a soluble calcium salt on warming; and neutral calcium paracasein (present in milk acted upon by rennet enzym) is coagulated at ordinary temperatures by soluble calcium salts. Neither neutral calcium casein nor neutral calcium paracasein is coagulated by rennet in the absence of soluble calcium salts.

(6) Neutral calcium casein, prepared by making a lime-water solution of free casein neutral to litmus, is an opalescent solution, free from any visible suspended particles; neutral calcium paracasein, prepared by making a lime-water solution of free paracasein neutral to litmus, is a clearly defined coagulum. In this respect only does there appear to be any marked difference in the behavior of the neutral calcium compounds of casein and paracasein. Here the difference is one rather of degree than of kind, since neutral calcium casein is coagulated by small amounts of soluble calcium salts on warming while neutral calcium paracasein is coagulated at lower temperatures by soluble calcium salts.
THE RELATION OF THE SALT-SOLUBLE PRODUCT IN CHEESE TO PARACASEIN.

It was from our work with cheese (American cheddar) that we gained the first suggestion which led us to investigate this field. It is desirable, therefore, that we should now, if possible, establish the relation between the salt-soluble, alcohol-soluble body obtained from cheese, which we were first led to regard as paracasein monolactate and the paracasein compounds we have prepared from milk and discussed in the preceding pages.

We have established the fact that fresh cheddar cheese contains a large percentage of its proteid in the form of a body soluble in warm five per cent. salt solution and in hot 50 per cent. alcohol. Weidmann\(^{21}\) dissolved from emmenthaler cheese by hot alcohol a substance called by him caseoglucin. Röse and Schulze\(^{22}\) made a quite extensive study of the properties of this body, but reached no definite conclusions as to whether it was an individual compound or what was its relation to cheese-ripening. Winterstein\(^{23}\) has studied the cleavage products of caseoglucin treated by concentrated hydrochloric acid and finds the proteid bases arginine and lysine present in proportions differing from the amounts obtained by Hart\(^{24}\) from casein. The results of his work cannot be regarded as conclusive, since variation of conditions in producing cleavage leads to quite variable results. A cleavage study made by us on a similar product suggests a close relation between casein and the salt-soluble product, which appears to be identical with caseoglucin. (Bulletin 214, p. 63, 1902.)

PREPARATION OF CHEESE EXTRACTS.

*Material used to furnish preparation.*—We used as the source of our material in making preparations for study a cheddar cheese one day old, which indicated by its stringing on a hot iron an abundance of salt-soluble substance. Portions of the cheese were ground in sand and extracted with water at 50 °C.


\(^{22}\) *Landwirth. Versuchs-Stat.*, 31:130 (1884-5).


The portion insoluble in water was then divided, part to be extracted with hot 50 per ct. alcohol and part with warm 5 per ct. salt solution.

*Preparation made by extraction with alcohol.*—The ground, water-extracted cheddar curd was treated with boiling 50 per ct. alcohol. The extract was filtered through paper on a hot-water funnel. The filtrate on cooling and standing deposited the extracted proteid in a rubber-like mass. This mass was ground up in water several times, filtered, washed with 95 per ct. alcohol and ether, and dried at 100° C. The product was a hard mass, difficult to pulverize. It contained 1.40 per ct. of ash.

*Preparation made by extraction with 5 per ct. solution of sodium chloride.*—The remaining portion of ground, water-extracted cheese was extracted with a 5 per ct. solution of sodium chloride at 60° C., filtered through paper twice and then precipitated with two volumes of 95 per ct. alcohol. On standing over night the precipitate separated. It was filtered, washed by decantation with water several times to remove the salt and finally thrown on a filter paper and washed until all sodium chloride was removed. The precipitate was then washed with alcohol and ether, and dried at 100° C. The product was easily powdered. It contained 1.37 per ct. of ash.

**Behavior of Salt-Soluble and Alcohol-Soluble Preparations Toward Lime Water.**

We made a study of the power of these two preparations to combine with calcium in comparison with base-free casein, in order to ascertain whether they were base-free or not. Our method was the following: In portions of 50 cc. each of lime water of known strength, we dissolved .5 gram each of base-free casein, of the salt-soluble preparation and of the alcohol-soluble preparation. These lime-water solutions of proteids were then treated with $\frac{1}{10}$ hydrochloric acid until the solution was neutral to phenolphthalein, with the following results:
TABLE IV.—POWER OF DIFFERENT PREPARATIONS TO COMBINE WITH CALCIUM.

<table>
<thead>
<tr>
<th></th>
<th>n-10 HCl required to neutralize</th>
<th>Difference of n-10 HCl between lime-water and solutions of proteids in lime-water</th>
<th>Equivalent to CaO combined with proteid</th>
<th>Per ct. of CaO combined with proteid</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 cc. lime-water</td>
<td>19.5</td>
<td>4</td>
<td>.01120</td>
<td>2.24</td>
</tr>
<tr>
<td>.5 gram of base-free casein dissolved in 50 cc. lime-water</td>
<td>15.5</td>
<td>.1</td>
<td>.01148</td>
<td>2.29</td>
</tr>
<tr>
<td>.5 gram of alcohol preparation dissolved in 50 cc. lime-water</td>
<td>15.4</td>
<td>4.1</td>
<td>.01093</td>
<td>2.19</td>
</tr>
<tr>
<td>.5 gram of salt-soluble preparation dissolved in 50 cc. lime-water</td>
<td>15.6</td>
<td>3.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

These results show that the salt-soluble and alcohol-soluble preparations made from cheese possessed the same power of combining with calcium as did casein or paracasein known to be base-free, and that they must therefore have been base-free proteids capable of forming a calcium combination equivalent to that of basic calcium casein or paracasein. These preparations from cheese appear therefore to be identical with base-free paracasein.

The calcium of the ash present was evidently not a part of the proteid molecule but was probably an impurity of calcium lactate or possibly this salt loosely combined with the proteid, appearing on ignition as calcium phosphate.

BEHAVIOR OF SALT-SOLUBLE AND ALCOHOL-SOLUBLE PREPARATIONS TOWARD ACIDS.

In determining the amount of acid that could combine with these salt-soluble and alcohol-soluble preparations made from cheese, we used the methods already employed in connection with casein and paracasein. We suspended .5 gram of proteid in water, added 10 cc. of $\frac{n}{100}$ hydrochloric acid, made the volume to 100 cc., filtered, and titrated the filtrate with $\frac{n}{100}$ sodium hydroxide, with phenolphthalein indicator. The results, figured on the basis of one gram of proteid, were as follows:
TABLE V.—ACID-COMBINING POWER OF DIFFERENT PREPARATIONS.

<table>
<thead>
<tr>
<th>Material used</th>
<th>Amount of acid used equal to n-100 HCl</th>
<th>Acid equal to n-100 HCl in filtrate</th>
<th>Acid equal to n-100 HCl in proteid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base-free casein</td>
<td>20.0</td>
<td>13.6</td>
<td>6.4</td>
</tr>
<tr>
<td>Alcohol-soluble preparation</td>
<td>20.0</td>
<td>13.6</td>
<td>6.4</td>
</tr>
<tr>
<td>Salt-soluble preparation</td>
<td>20.0</td>
<td>14.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

In respect to the power of combining with acids, these results indicate that the alcohol-soluble and salt-soluble preparations made from cheese are essentially the same compounds as casein and paracasein, that is, that they are essentially base-free paracasein.

THE NOMENCLATURE OF CASEIN AND PARACASEIN AND THEIR COMPOUNDS.

From the work presented in the preceding pages, there is reason to believe that there exist the following bodies, representing milk-casein and paracasein and the compounds formed by them:

Casein, the base-free proteid or free casein.

Basic calcium casein, containing free casein combined with about 2.40 per ct. of CaO.

Neutral calcium casein, calcium casein containing about 1.50 per ct. of CaO in combination, probably identical with casein as it exists in cows' milk.

Casein salts of acids, compounds existing as precipitates and formed by combination of the free proteid with acid.

Paracasein, the base-free proteid formed by treating calcium casein with rennet enzym.

Basic calcium paracasein, containing free paracasein combined with about 2.40 per ct. of CaO.

Neutral calcium paracasein, calcium paracasein containing about 1.50 per ct. of CaO in combination, probably identical with the uncoagulated body present in cows' milk treated with rennet enzym in the absence of soluble calcium salts.

Paracasein salts of acids, compounds existing as precipitates and formed by combination of the free paracasein with acid.
In most of the literature on the subject, the word casein is used indiscriminately to mean milk-casein, free casein, or those casein salts formed by acid precipitation. In many cases it is used comprehensively to include all the proteids in cows' milk. A similar state of confusion exists in regard to the use of the word paracasein. It would therefore seem pertinent to make the following suggestions, tentatively at least, in regard to the nomenclature of these compounds:

1. That the word casein be applied only to the free proteid, that is, the base-free casein.
2. That the compound existing in cows' milk and commonly called casein be called calcium casein.
3. That the casein compound containing about 2.40 per cent. of CaO be called basic calcium casein.
4. That a compound formed by precipitation and combination with an acid be called a casein salt of the acid used.
5. That the same nomenclature be applied to the corresponding paracasein bodies, simply substituting the word paracasein for casein, with the following addition: Calcium paracasein should be applied to the uncoagulated substance produced in milk by rennet enzym, while the coagulum of this substance caused by soluble calcium salts should be called coagulated calcium paracasein.

THE RELATION OF THE RESULTS TO CERTAIN CHANGES TAKING PLACE DURING THE CHEESE-MAKING PROCESS.

In the manufacture of cheddar and similar types of cheese, after the addition of rennet enzym, and coagulation, there takes place a progressive change, resulting in the production of increased amounts of a proteid soluble in warm 5 per cent. solution of sodium chloride. This product may amount in fresh cheese to 75 to 80 per cent. of the proteids present. We were led by our former work to interpret these facts as follows: Lactic acid, formed by the lactic fermentation of milk-sugar, combines with the paracasein of the curd, forming paracasein mono-lactate, insoluble in water but soluble in warm dilute salt solution and in
hot 50 per ct. alcohol. In the light of the results of our more recent work, this interpretation must be modified and the observed facts appear to be explained correctly in the following manner: The coagulum following the addition of rennet enzym to milk is calcium paracasein, either mixed or loosely combined with soluble calcium salts. While lactic acid is being formed in the cheese-making process, it combines with the calcium of the calcium paracasein, forming free paracasein and calcium lactate. The conditions of manufacture are so controlled that normally not enough acid is produced to convert all the calcium para-
casein to base-free paracasein. The proteids of the curd are therefore a mixture, in varying proportions, of calcium paracasein and free paracasein. It is the free paracasein that is soluble in warm 5 per ct. salt solution and in hot 50 per ct. alcohol; and it is this body that has the characteristic property of being drawn out in fine, silky threads, when touched with a hot iron. It is the free paracasein that imparts to cheese curd its peculiar plastic and ductile properties, exhibited in the process known as "packing" or "matting." It is the free paracasein, therefore, that appears to be the body in which begin to take place the various chemical changes grouped under the general term cheese-ripening.

When, in the process of cheese manufacture, an excess of lactic acid is produced, as .7 or .8 per ct., we have the product familiarly known as cottage or Dutch cheese. This product is of a loose, granular structure and is insoluble in warm salt solution. In this case all the calcium of the calcium paracasein com-
bines with lactic acid, after which additional amounts of free lactic acid formed unite in a loose combination with the free paracasein, producing paracasein lactate, which differs from free paracasein in a most marked manner in respect to its solubilities in dilute salt solution and hot alcohol, its plasticity and its ability to form fine strings when heated.