Some Investigations on the Phenomena of "Clot" Formations. Part I.—*On the Clotting of Milk.*

By S. B. Schryver.

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(From the Research Institute of the Cancer Hospital.)

Introduction.

During the course of some researches on the bile acids, the observation was made that sodium cholate in 1-per-cent. solution reacts with calcium salts in a characteristic manner. On first addition of the salt solution, the mixture remains clear; on gentle warming, however, it solidifies to form an irreversible gel, the rate of formation of which, other factors being the same, is dependent on the temperature. A series of experiments was carried out to ascertain what influence the concentrations of the various calcium salts exerted on the time required for "clot" formation. These experiments were carried out at a temperature of 50° C. It was found as a result, that the calcium salts, as regards their behaviour to sodium cholate, could be divided into two classes. In the first class are included those salts in the presence of which the clotting time diminishes as the concentration of their solutions is progressively increased. In the second class of salts, the clotting time also diminishes as the concentration is increased, but only up to a certain optimal point; further concentration beyond this point increases the time of clot formation, or even inhibits it entirely. The salts of the first class can be distinguished from those of the second, in that the former increase the surface tension of water (measured against air), whereas the latter cause a diminution of this constant. The greater the diminution of the surface tension caused by salts of the second class, the smaller the range of concentration of the salt solution within which complete clot formation is possible. The actual chemical nature of the clot has not yet been ascertained. It is possibly either calcium cholate or the free acid (formed by hydrolysis of the calcium salt); in either case it is obviously a heavily hydrated aggregate, the formation of which is inhibited by the presence of readily adsorbed substances; the more easily such substances lower the surface tension of water, the more readily are they adsorbed from aqueous solution, and the

greater their inhibitory action on the formation of aggregates by the larger molecules contained in the system.*

It is proposed to deal with the subject of the cholate clot in a subsequent publication, but a preliminary account of certain experiments is given in this place, as the results obtained have given rise to suggestions as to the mechanism of a phenomenon of far greater general interest, viz., the clotting of milk by rennet.

From the fact that an aggregation, commonly known as a "clot," can be inhibited by the presence of simple adsorbable substances in a system, the conception arose that in milk the substances necessary for the formation of the clot already pre-exist, but owing to their adsorption of simpler molecules from the system, they do not aggregate, but remain in a state of dispersion. It was thought that a ferment, for which the disperse phase acts as a substrate, could clear the surface of the colloidal particles of adsorbed substances, and thus allow their aggregation to take place; in other words, the ferment could act the part of a scavenger.

If the view here advanced is correct, it should be possible to produce clot formation from a milk protein by the action of calcium salt alone, in the absence of rennet. Indications of such a possibility are afforded by the experiments published more than 20 years ago by Ringer.†

A more or less similar conception underlies the experiments recently published by Hedin and his pupils. The former has shown that ferments, such as rennet, are adsorbed by charcoal, and in this adsorbed state do not exert their full activity; if to such a combination another readily adsorbable substance, such as saponin, is added, the ferment can be freed from its combination with the charcoal and its activity can be partly or wholly restored.‡

Whatever chemical process is involved in the actual clot formation in milk, it is a fact, which is illustrated by various experiments in this paper, that when aggregation is prevented by adsorbable substances, the inhibitory action of the latter appears to be antagonised by the addition of an appropriate enzyme.

* For other examples of salt action, see Schryver, "Investigations dealing with the State of Aggregation of Matter, Parts I–III," 'Roy. Soc. Proc.,' 1910, B, vol. 83, pp. 96–123.

† 'Journ. Physiol.,' 1890, vol. 2, p. 464.

‡ Hedin, 'Zeitsch. physiol. Chem.,' 1912, vol. 82, p. 175; and Jahnson-Blohm, *ibid.*, p. 178.

The Lability of Caseinogen.

The first experiments on milk clotting were directed towards a preparation of a standard caseinogen solution, upon which the action of alkalis could be quantitatively studied. For the preliminary investigations, two different samples were employed, viz., a commercial preparation made by the "Rhenania" factory of Aix-la-Chapelle, and a preparation made in the laboratory by a slight modification of Hammarsten's process.

The Solubility of Caseinogen in Alkalis.

It has been long known that caseinogen can dissolve in alkalis to give rise to highly acid solutions of what are presumably acid salts. The degree of solubility in alkaline solutions was employed throughout these researches to characterise the various preparations.

In the determination of this factor, no pretence can be made to the accuracy which can be attained in the estimation of the solubility of crystalline substances, owing to various inherent experimental difficulties. The alkaline solution most generally employed was that of saturated calcium hydroxide diluted with its own volume of freshly boiled water ($\frac{1}{2}$ sat. $Ca(OH)_2$). An excess of the caseinogen preparation (usually 1·5–2 grm.) was mixed with 20–25 c.c. of the lime water solution in a bottle, which was then placed over night (usually about 17 hours) on a rotating axis in a thermostat kept at 21·5° C. At the end of this period a saturated solution of caseinogen in lime water containing the excess of undissolved caseinogen was obtained. This mixture could not be directly filtered in the thermostat, as the pores of the filter soon become clogged. In order to get a filtrate, the mixture had to be submitted to prolonged centrifugalisation. An attempt was made to work in every case under as nearly as possible the same conditions. The mixture was consequently always centrifuged for two hours at a speed of 3500 revolutions per minute. After this treatment, the supernatant fluid could be readily filtered through small folded filters. The defects of this method are obvious. It is impossible to work under absolutely constant conditions, and the liquid becomes slightly warm as the result of the centrifugalisation. As there is evidence that the calcium salt of caseinogen undergoes hydrolysis readily when solutions diluter than half-saturated lime water are employed as a solvent, it can be readily understood that errors can arise from the want of constancy of temperature throughout the whole experiment due to the impossibility, with the laboratory appliances available, of carrying out all investigations at exactly the same temperature. Furthermore, the alkaline solutions employed are very dilute, and a relatively small error in standardising

such solutions can cause a relatively large error in the solubility determination. Better results would also probably have been obtained had it been feasible to avoid the use of glass vessels. In spite, however, of all these known inaccuracies, the deviations from the absolutely correct figures are so small compared with the changes produced by submitting caseinogen to various treatments, that the lack of rigidity in the experiments does not materially affect the conclusions drawn from the results; it accounts for the small irregularities to be noticed in the various tables of results.

The nitrogen was estimated in 5 c.c. of the filtrate by Kjeldahl's method. The number of cubic centimetres of N/10 acid required to neutralise the ammonia produced will, throughout this paper, for the sake of brevity, be designated simply the solubility of the preparation.

Differences in the Solubility in Alkalis of Various Preparations.

The earliest experiments indicated that great differences existed between the various preparations employed.

A sample of a Rhenania caseinogen showed a solubility in $\frac{1}{2}$ sat. $Ca(OH)_2$ of 5·1, whereas the solubility of the preparation prepared by the modification of Hammarsten's method mentioned above was 21·2. Sodium hydroxide solutions dissolve approximately the same amount of substance as the equimolar (and not equinormal) calcium hydroxide solutions. In this respect caseinogen behaves like other proteins, such as edestin and globulin.

Attempts were then made to account for the differences in the behaviour of the various preparations. A series of products was made from milk, by the method already described, but instead of purifying the precipitated caseinogen by dissolving it in sodium hydroxide, it was treated with other alkalis (normal solutions), from the solutions in which it was precipitated by acetic acid; the procedure was exactly the same as when sodium hydroxide was employed.

No marked difference was found in the solubility of the various preparations thus obtained.

Nevertheless, the numbers found are of quite a different order to the solubility of the Rhenania preparation (5·1). The effect of the treatment of the Rhenania caseinogen by alkali was next investigated. This was dissolved in normal alkali, reprecipitated, and treated by the general routine process (alcohol, ether, etc.). The preparation thus obtained was now much more soluble in $\frac{1}{2}$ sat. $Ca(OH)_2$, viz., 21·1, *i.e.* it was of the same order as that of the preparations made in the laboratory.

Attempts were next made to ascertain the reason of the great differences

in the behaviour of the various samples. The action of water at higher temperatures was first investigated.

A sample of caseinogen, freshly precipitated, was treated with alcohol and ether, and then air dried. (Solubility, 21·5.) It was then warmed with water for half an hour at 70°, which caused it to form at first a pasty mass, which became more granular as the heating was continued. It was then treated with alcohol and ether and air-dried. The solubility in $\frac{1}{2}$ sat. $Ca(OH)_2$ was now 10·9, or only a little more than half that of the original preparation. On redissolving the heated product in sodium hydroxide and reprecipitating, a preparation was obtained with the solubility 25·3. Another sample of the heated product was "purified" by solution in ammonia, and the preparation thus produced had a solubility of 22·3. A third sample was "purified" by dissolving in calcium hydroxide. The caseinogen was only very partially precipitated by acetic acid from this solution, and the filtration of the precipitate through paper could only be effected with difficulty. The solubility of this preparation was 12·6.

It is therefore obvious from these experiments that caseinogen, on treatment with hot water, is converted into a product which is considerably less soluble than the original substance in lime water, but which, on "purification" by solution in caustic alkalis and reprecipitation by acids, is reconverted into the substance from which it was formed.

The solubility of heated and unheated preparations in various strengths of alkaline solutions was next determined, and a comparison of the solution capacities of calcium and sodium hydroxide was made.

Saturated $Ca(OH)_2$ solution requires for neutralisation 4·3 c.c. N/10 acid.

An equimolar Na(OH) solution requires for neutralisation $\frac{4{\cdot}3}{2}$ c.c. N/10 acid.

In the following table the figures indicate the number of cubic centimetres of N/10 acid necessary to neutralise the ammonia produced by the Kjeldahlisation of 5 c.c. of the solution. (For method of determining the solubilities and sources of error, see pp. 462–3.)

Original preparation.		Heated preparation.	
Solubility in $Ca(OH)_2$ solutions.	Solubility in NaOH solutions equimolar with	Solubility in $Ca(OH)_2$ solutions.	Solubility in NaOH solutions equimolar with
Saturated, 46·5 $\frac{1}{2}$ saturated, 22·8 $\frac{1}{4}$ saturated, 6·6	Saturated $Ca(OH)_2$, 41·4 $\frac{1}{2}$ saturated $Ca(OH)_2$, 20·3 $\frac{1}{4}$ saturated $Ca(OH)_2$, 10·0	Saturated, 23·9 $\frac{1}{2}$ saturated, 11·9 $\frac{1}{4}$ saturated, 4·3	Saturated $Ca(OH)_2$, 36·6 $\frac{1}{2}$ saturated $Ca(OH)_2$, 16·4 $\frac{1}{4}$ saturated $Ca(OH)_2$, 7·6

It is necessary to call attention to two facts which are indicated in the above table, viz.:—

(1) Whereas saturated lime water dissolves only a very little more than twice as much of the preparations dissolved by the half-saturated solution, the latter dissolves very appreciably more than twice the amount dissolved by lime water of quarter saturation. A similar result has been obtained several times, and it indicates that when the strength of solution is below that equivalent to half saturation, hydrolytic dissociation of the calcium salt takes place. A similar phenomenon is not noticed in the case of the sodium salt.

(2) The solution capacity of sodium hydroxide for the heated preparation is considerably larger than that of the equimolar solution, more especially in the highest concentration. This is in accordance with the fact that caustic alkalis reconvert the product obtained by heat change into its original form.

In order to produce the changes in caseinogen discussed above water is necessary. A preparation of caseinogen shows no change in its solubility in calcium hydroxide after boiling for half an hour with alcohol.

A Systematic Examination of the Action of Water on Caseinogen.

For the purpose of a more detailed investigation on the action of water, samples of caseinogen, each of 5 grm. weight, which had been "purified" twice by solution in sodium hydroxide and reprecipitation with acid (but without any special precautions as to temperature or time of contact with reagents) were warmed at different temperatures (in thermostat) and for different periods with 10 times the weight of water. At the end of the treatment the water was poured off, and the samples were then treated with alcohol and ether, and air-dried. On treatment with alcohol those which had been submitted to higher temperatures, and which on first heating became pasty, were converted into a hard granular mass, and were finely ground with alcohol in a mortar. In the following table the solubilities in $\frac{1}{2}$ sat. $Ca(OH)_2$ of the different preparations are given:—

Solubility of Original Preparation, 22.

Temperatures	37°.	50°.	75°.	100°.
Time of heating—				
½ hour	18·8	16·3	14·4	13·0
1 „	17·5	15·7	11·0	10·8
2 hours.....	15·8	13·3	10·9	—
5 „	13·7	12·5	10·7	7·4
10 „	11·4	11·5	10·8	6·9
26½ „	11·6	10·1	6·8	4·2

From the above table it will be observed that caseinogen is changed by simply heating with water at incubator temperature. The change proceeds until a solubility denoted by the number 11 is attained. When this point is attained further change appears to be very slow. At higher temperatures, however, further changes are produced, and the caseinogen is converted into a product which is no longer soluble in excess of sodium hydroxide. The products obtained by the action of water at higher temperatures have not been further investigated.

In view of the fact that excess of casinogen preparation was always used in the solubility estimations, and that consequently the saturated solutions of caseinogenate were kept for some time in agitation with this excess, experiments were carried out to ascertain how far adsorption phenomena influenced the final equilibrium. For this purpose, 20 c.c. of saturated solutions of the original preparation and a preparation which had been heated for three-quarters of an hour with water at 75°, in $\frac{1}{2}$ sat. $Ca(OH)_2$, were rotated with $1\frac{1}{2}$ grm. of the various heated products for 17 hours. The mixtures were then centrifuged and the nitrogen was estimated in 5 c.c. of the filtered supernatant fluid. It was found that a small amount of the caseinogenate was thus removed from solution. Thus in the case of the unheated caseinogenate 5 c.c. required, after Kjeldahlisation, about 20 c.c. N/10 acid to neutralise the ammonia produced. Of the same preparation, after rotating with various samples of heated products, 5 c.c. required 17·4–18·2 c.c. In the case of the heated product, 5 c.c. of the caseinogenate were equivalent to 10·0 c.c. of acid, and after rotation with the heated products, to 8–9 c.c. of acid. There was no marked difference in the adsorption capacities of the different heated products. Adsorption phenomena have therefore but little influence on the comparative values of the numbers given in the above table.

The Preparation of "Natural" Caseinogen.

As the above series of experiments have demonstrated the great lability of caseinogen, which, under the influence of water, is readily converted into one or more "metacaseinogens," experiments were next directed to ascertain whether the natural caseinogen undergoes change during the course of its preparation by Hammarsten's method (or modifications of the same).

Efforts were directed towards obtaining a product which should remain for as short a time as possible in contact with the various reagents employed.

To accomplish this end a caseinogen was prepared in the following manner:—Four litres of skimmed milk were diluted with 16 litres of water, and to this mixture 20 c.c. of glacial acetic acid dissolved in 400 c.c. of water were added with brisk agitation. After not more than two or three

minutes, when the caseinogen had settled at the bottom of the precipitating vessels, the supernatant liquid was syphoned off, this process being accomplished in as short a time as possible. The precipitate was then washed twice by decantation with several times its volume of ice-cold water. It was then treated with graded strengths of alcohol, up to absolute alcohol, then with ether, and finally air-dried. A very fine light power was thus obtained, which was considerably more soluble in $\frac{1}{2}$ sat. $Ca(OH)_2$ than any of the preparations obtained by the ordinary Hammarsten method, when the precautions described above were not observed.

Whereas the solubility of the Hammarsten preparations in lime water varied as a rule (and the reason of these variations is now obvious) between 20 and 26, that of the new preparation was about 35. The solubilities of a large number of preparations obtained by the new method were determined; the majority of them showed a solubility in $\frac{1}{2}$ sat. $Ca(OH)_2$ which varied but slightly from that given.

Furthermore, the solutions thus obtained both in $\frac{1}{2}$ sat. $Ca(OH)_2$ and the equimolar sodium hydroxide solution, especially that in lime water, had an opaque milky appearance which retained the opacity even after dilution with several volumes of water, which is in marked contrast to the opalescent solutions obtained with the ordinary commercial preparations.

The Lability of the "Natural" Caseinogen.

The factors influencing the changes in the "natural" caseinogen were next investigated.

Effect of Acids.

Ten-gramme samples of the "natural" caseinogen were allowed to stand with 100 c.c. of a 0·1-per-cent. acetic acid solution, which is about the strength of the acid in which the original precipitate is produced. The samples were allowed to stand for varying times with the acid, then filtered, washed with water, and repeatedly with alcohol, to remove the last traces of acid. They were finally washed with ether and air-dried. The following numbers indicate the solubilities in $\frac{1}{2}$ sat. $Ca(OH)_2$ after varying periods of contact with 1/1000 acetic acid:—

Original preparation	35·0
After 1 hour with acid.....	27·3
„ 2 hours „	19·3
„ 6 „ „	15·5
„ 24 „ „	11·4

The "natural" caseinogen is therefore very unstable in the presence of acids.

This change in the caseinogen is not due to a ferment carried down with it when it is precipitated from milk, as a product of high solubility in alkali is also obtained when the milk is boiled before the precipitation of the caseinogen. Some preliminary experiments, which need not be given in detail here, indicate that substances present in the milk serum protect the caseinogen from change, when treated with water.

Action of Water on "Natural" Caseinogen.

The action of water on "natural" caseinogen at various temperatures was also investigated in some detail. The method of experiment was exactly the same as that employed in the researches on the action of water on caseinogen prepared by Hammarsten's method (see p. 465). The result of the determinations of the solubility in $\frac{1}{2}$ sat. $Ca(OH)_2$ of the various products are indicated in the following table :—

Solubility of Original Preparation, 35.

Temperatures	37°.	75°.	100°.
Time of heating—			
½ hour	16·8	17·6	14·5
1 „	17·2	18·5	13·4
2 hours	14·1	13·8	11·2
5 „	14·7	11·7	9·0
10 „	13·4	11·8	6·0
26½ „	11·5	10·8	4·9

The changes at 51° did not differ materially from those at 37°. It will be observed that up to 75° the temperature has but little effect on the rate of change. Within the first half-hour, however, the caseinogen, even at 37°, breaks down into a product with only about half the solubility of the original preparation in $\frac{1}{2}$ sat. $Ca(OH)_2$. At ordinary room temperature the change is very slow, a preparation left in contact with water for 24 hours having its solubility reduced only to about 30. It is not at present possible to formulate the chemical changes which have been described above. It seems, however, feasible to assume that they are produced by the scission or addition of the elements of water. It is advisable to recall the fact that water is necessary to produce these changes, and the caseinogen itself remains unaltered on boiling with alcohol. If the above conception is correct the presumably more complex and more soluble caseinogen is a poly-acid which first undergoes hydrolysis into a simpler

acid, from which, on heating, the elements of water are removed from contiguous hydroxyl groups. On treatment with caustic alkalis, and acidification of the alkaline solution, these elements are added again to the molecule, which is thereby enabled to again form the poly-acid. The relationship, if this view is correct, is similar to that existing between a pyrophosphate and a metaphosphate, a conception which is not improbable when it is remembered that caseinogen is a derivative of phosphoric acid.

Physically there is a marked difference between the saturated solutions (*i.e.* as regards caseinogen) of the calcium salts (containing the same amount of calcium) of caseinogen and metacaseinogen. The former are white opaque solutions which retain their opacity even after considerable dilution, whereas the latter (produced by the prolonged action of water at 37° on natural caseinogen) are translucent, although opalescent.

It may be recalled here that these two forms are readily convertible, one into the other. As might be expected, the nitrogen–phosphorus ratios remain constant. The analyses of the various products are not quoted in this paper. They are also neutralised by the same amount of sodium hydroxide.

The Action of Calcium Salts on Solutions of "Natural" Caseinogenates.

Throughout all the following experiments a saturated solution of "natural" caseinogen in $\frac{1}{2}$ sat. $Ca(OH)_2$ (10 c.c. = 2·15 c.c. N/10 acid) and equimolar carbonate-free sodium hydroxide (20 c.c. = 2·15 c.c. N/10 acid) have been employed. The solutions were always prepared by gentle rotation for 17 hours in a thermostat of 4 grm. of caseinogen with 40 c.c. of the alkaline solution. The mixture was afterwards centrifuged for two hours at a speed of 3500 revolutions per minute, and the supernatant fluid decanted off.

Action of Calcium Chloride Solutions on Sodium Caseinogenate Solutions.—When the caseinogenate solutions are treated with calcium chloride, a precipitate is formed, but only within certain limits of the concentration of the calcium salt. The reactions form therefore an "irregular series," a phenomenon which is not uncommon when a reacting substance is a complex colloid.*

In the following experiment 10 c.c. of the sodium caseinogenate solution were diluted with 10 c.c. of calcium chloride solutions of varying strengths. After standing, the mixtures were filtered through folded filter-papers, and the nitrogen was estimated in 10 c.c. of the filtrate. The results are given in the following table:—

* These reactions are discussed in some detail in a footnote to a former paper ('Roy. Soc. Proc.,' 1910, B, vol. 83, pp. 97 and 98).

	N in filtrate.	Percentage precipitated.
10 c.c. caseinogenate solution—		
+ 10 c.c. water	28·0	0
+ 10 c.c. N/50 $CaCl_2$	27·4	2·2
+ 10 c.c. N/25 „	1·2	95·7
+ 10 c.c. N/20 „	1·0	96·4
+ 10 c.c. N/15 „	1·2	95·7
+ 10 c.c. N/10 „	1·8	93·6
+ 10 c.c. 3N/20 „	3·0	89·3
+ 10 c.c. N/4 „	28·0	0
+ 10 c.c. N/2 „	28·0	0

It will be seen from the above table that nearly complete precipitation takes place only when the concentration of the calcium salt in the mixture lies between N/50 and 3N/20. When the concentration reaches N/4 no precipitation takes place. If, however, a drop of rennet extract is added to the mixture containing the higher concentrations of the calcium salt, precipitation takes place after the interval of a few minutes. Without such addition, no precipitation occurs even after prolonged standing.

The product obtained by the above described reaction appears to be a calcium salt produced by double decomposition between the sodium caseinogenate and calcium chloride. For analysis, the precipitate was washed with 50-per-cent. alcohol, till free from chloride, and then with alcohol and ether, and air-dried. It is a true precipitate which lacks the characteristic physical properties of a clot, which are described below.

Other Methods of Inhibiting Precipitation.—The formation of the precipitate can also be inhibited when the optimal proportions of calcium salt are present by the addition of other substances. In view of the conceptions advanced in the introduction to this paper, it was of importance to investigate more especially the inhibitory action of the substances contained in the milk serum. This was prepared by the addition of sufficient rennet to skimmed milk. As soon as the formation of the clot was complete it was broken up, and the serum was filtered off through muslin, boiled, and again filtered from the protein coagulum. In addition to the action of milk serum, the inhibitory action of Witte's peptone and of glycine were investigated.

Some of the results are recorded in the following table:—

1 c.c. caseinogenate solution—

+0·1 c.c. N $CaCl_2$ solution	+	0·9 c.c. water	.....		Immediate precipitate.
„	„	+ 0·7	„	+ 0·2 c.c. serum	Almost immediate precipitate.
„	„	+ 0·5	„	+ 0·4 „	Precipitate nearly complete.
„	„	+ 0·3	„	+ 0·6 „	Precipitate incomplete.
„	„	+ 0·1	„	+ 0·8 „	No precipitate.*
„	„			+ 0·9 „	„ *

In the cases where no precipitate was formed in the cold (indicated by an asterisk, *), it came on warming gently (in the hand), but disappeared again on cooling. A reversible reaction apparently takes place. If, however, rennet is added, the precipitate is rendered permanent. The same phenomena were observed in a further series of similar experiments, in which, in one case, a 5-per-cent. solution of Witte's peptone, and, in another case, 10-per-cent. solution of glycine, were employed instead of milk serum.

A further series of experiments was also carried out, in which only half the concentration of calcium salt was present, and the results are as follows:—

1 c.c. caseinogenate solution—

+0·1 c.c. N/2 $CaCl_2$ solution	+	0·9 c.c. water	.....		Immediate precipitate.
„	„	+ 0·7	„	+ 0·2 c.c. serum	Turbidity, precipitate on warming which is rendered permanent by rennet.
„	„	+ 0·5	„	+ 0·4 „	„ „
„	„	+ 0·3	„	+ 0·6 „	Precipitate only on warming + rennet.
„	„	+ 0·1	„	+ 0·8 „	No precipitate.
„	„			+ 0·9 „	No precipitate even on warming and when rennet is present.

Similar results were obtained in experiments in the presence of Witte's peptone and glycine. It will be seen from the above tables that the conditions necessary for the production of a permanent precipitate are somewhat complex, and depend on the relative quantities of inhibitory substances and calcium salt present. They require investigation in greater detail. Whatever interpretation may be given to the particular action, it will be seen that the ferment can produce an aggregation in a system where it is otherwise inhibited by the presence of simple non-colloidal substances, although, perhaps, the interpretation of its action is not as simple as that suggested in the introduction of this paper. This point will, however, receive attention later, in the discussion of the mechanism of clot formation.

Action of Calcium Chloride on the Solution of "Natural" Calcium Caseinogenate. Production of Clot without Intervention of Rennet.

To portions of 10 c.c. of a saturated solution of caseinogen in $\frac{1}{2}$ sat. $Ca(OH)_2$, in a series of test-tubes, were added 10 c.c. of calcium chloride solutions of the following concentrations: N/25, N/20, N/15, N/10, 3N/20, N/5, N/4, N/2. On allowing these mixtures to stand at room temperature no change was observed. On putting the tubes in an incubator, however, the solutions all clotted when the concentrations of the calcium chloride added did not exceed 3N/20. When the concentration was N/5, an incomplete clot formed, but above this limit the liquid remained turbid, and gave no indications of a clot even after prolonged warming in an incubator. The addition of rennet in these cases produced, however, an aggregation even after a short interval. The clots produced all shrank after standing, leaving, when clotting had been complete, a clear supernatant fluid. They possessed, furthermore, a characteristic physical property, which they share in common with the clot produced directly by the action of rennet on milk, and which distinguishes them from precipitated caseinogen, for whereas the latter, on treatment with alcohol, does not alter in the general appearance, the former yield an indiarubber-like mass, which, on further treatment with alcohol, is converted into hard granular products, which can be pulverised only with some difficulty.*

Clot formation can be inhibited not only by excess of the calcium salt, but also by milk serum and solutions of Witte's peptone and glycine. These experiments were carried out with N/25 $CaCl_2$ in milk serum concentrated to half the original bulk *in vacuo*, in milk serum in its original concentration, in 5-per-cent. Witte's peptone solution, and in 10-per cent. glycine; 5 c.c. of these various solutions were added to 5 c.c. of the caseinogenate solution, and the mixtures were allowed to stand for one hour at 37°. At the end of the period no trace of clotting had taken place. The addition of one drop of rennet solution caused the clot to form in less than five minutes. The solutions containing the peptone clotted somewhat more slowly, and the clot formed differed somewhat in character from the other clots, for whereas the latter were firm and shrank in the characteristic manner, the former was more liquid and shrank to a heavy oil.

The behaviour of solutions of calcium and sodium caseinogenate towards calcium chloride were in most respects similar, aggregation taking place within only certain definite limits of concentration and being inhibited by

* Ringer (*loc. cit.*), by the action of calcium chloride on a solution of calcium "caseinogenate," produced a curdy deposit in the cold. This reaction differs from the one described above. Ringer's "caseinogenate" solution behaves, in fact, as a "metacaseinogenate" solution, the action of calcium chloride on which is described on p. 473.

the presence of milk serum, Witte's peptone, and glycine. But whereas the sodium caseinogenate formed precipitates immediately in the cold, which precipitates were not altered in appearance by treatment with alcohol, the calcium salt reacted only on slight warming (the temperature of 25° is sufficient to produce clot formation), and yielded not a precipitate but an aggregation with the characteristic physical appearance and properties of an ordinary milk clot. In both cases rennet could produce aggregation which had been inhibited by the presence of simple adsorbable substances.

Clot formation could also be produced by the action of strontium and barium chlorides. The former acted quantitatively, like calcium chloride, but the latter had a greater range of action, producing a clot when the calcium caseinogenate solutions were diluted with equal volumes of barium chloride solutions in concentrations varying from N/50 to N/5. Even when the concentration reached N/4 a nearly complete clot was formed. Sodium chloride, when added in concentrations of N/50 to 5N (nearly saturated solution), produced no action.

Action of Calcium Chloride on Calcium Metacaseinogenate.

A preparation of metacaseinogen was made by warming "natural" caseinogen for two days with water at 37°. The action of calcium chloride solutions on a saturated solution of this preparation in $\frac{1}{2}$ sat. $Ca(OH)_2$ was investigated. Again an "irregular series" of reactions was produced, but no clots. In the optimal concentrations of calcium chloride only a very partial precipitation was produced, but no precipitate formed (only an opaque fluid) in the presence of excess of this reagent. In these cases, the addition of rennet also produced a precipitate. The general results are indicated in the following table, which illustrates the marked contrast between the behaviour of caseinogen and metacaseinogen :—

	N in 10 c.c. of filtrate.	Percentage precipitated.
10 c.c. metacaseinogenate solution—		
+ 10 c.c. N/50 $CaCl_2$	10·4	0
+ 10 c.c. N/25 „	5·5	47·1
+ 10 c.c. N/20 „	3·5	66·3
+ 10 c.c. N/15 „	3·0	71·1
+ 10 c.c. N/10 „	3·8	63·4
+ 10 c.c. N/5 „	6·9	33·6
+ 10 c.c. N/4 „	10·4	0
+ 10 c.c. N/2 „	10·4	0

Other Methods of Obtaining Clots. The General Character of Clot Formation.

The experiments already quoted seem at first sight to fully confirm the theory as to the general action of ferments and inhibitory substances indicated in the introduction. They do not, however, indicate the actual chemical process of clot formation, and the whole subject is somewhat complicated by the fact that caseinogen itself is very labile. Further investigations were therefore necessary to ascertain the nature of the chemical processes involved in clot formation, and to determine whether the change of caseinogen was a necessary preliminary, and whether calcium or any other alkaline earth was an essential constituent of the clot-producing system.

During the course of these additional researches, it was found that it was possible to produce a clot by other methods.

It was found that the calcium caseinogenate solution alone, and without the addition of another calcium salt, could produce a clot on the addition of rennet. This clot formation, which can take place at room temperature, was, however, completely inhibited when the caseinogenate solution was diluted with a equal volume of milk serum, *i.e.* when the milk serum constituents were in only half the concentration in which they exist in milk. In the presence of such quantities of inhibitory solutions, the presence of an additional calcium salt is necessary to produce a clot. As caseinogen can be converted into metacaseinogen by water, it is conceivable that a similar reaction would take place more readily in the presence of rennet. In this case calcium caseinogenate should form a mixture of free metacaseinogen and calcium metacaseinogenate and there would then exist in the system more metacaseinogen than is necessary to saturate all the calcium present, for, as has already been shown above, a saturated solution of this substance in $\frac{1}{2}$ sat. $Ca(OH)_2$ contains only about a third of the amount of nitrogen contained in a corresponding solution of caseinogen. As a matter of fact there is evidence, which is given in greater detail below, that the clot formed in the presence of rennet is produced from metacaseinogen. It is conceivable, therefore, that the clots are formed from the free caseinogen or metacaseinogen directly and not from the calcium salts.

This supposition is also confirmed by other facts. By reference to the table given on p. 464 it will be seen that $\frac{1}{4}$ saturated lime water dissolves less than half the amount of caseinogen or metacaseinogen dissolved by half-saturated lime water, whereas completely saturated lime water dissolves only very little more than double the amount dissolved by the $\frac{1}{2}$ sat. $Ca(OH)_2$. Repeated experiments on the solubility of natural caseinogen in $\frac{1}{4}$ sat. $Ca(OH)_2$ gave solubility numbers varying between 8 and 11 instead

of the number 17 or 18, which would have been expected, had the solubility been one-half of that in ½ sat. $Ca(OH)_2$. As these figures indicate hydrolytic dissociation of the calcium salt in low dilutions, it is not unreasonable to suppose that the saturated solution of caseinogen in ½ sat. $Ca(OH)_2$ undergoes a similar hydrolysis when the temperature is raised.* Now attention has been called to the fact that calcium chloride does not produce a clot with calcium caseinogenate at room temperature, but only when the mixture is slightly warmed (*e.g.* to 25°). If, however, the calcium caseinogenate is first treated with carbon dioxide gas, which produces by itself no precipitate, the subsequent addition of calcium chloride rapidly produces a clot in the cold. Finally, it is possible to produce a clot from sodium caseinogenate solutions in the absence of calcium salts. Rennet by itself produces no clot from such solutions; if, however, they are treated first with carbon dioxide, the addition of the ferment solution causes clot production after a short interval, even in the cold. There is evidence that the sodium salt does not readily undergo hydrolytic dissociation (see table on p. 464). Carbon dioxide can apparently decompose the sodium salt and set free sufficient free caseinogen to allow the clotting process to take place.

Saturated solutions of metacaseinogen (prepared by the treatment of "natural" caseinogen with water for 24 hours at 37°) in ½ sat. $Ca(OH)_2$ do not clot on the addition of rennet, and yield only a very faint precipitate on addition of the ferment after previous treatment with carbon dioxide. The action of calcium chloride on these solutions has been already described. By means of these reactions, metacaseinogen can be readily distinguished from caseinogen.

Caseinogen, after solution in alkalis and reprecipitation with acetic acid, can yield solutions in ½ sat. $Ca(OH)_2$, which clot on the addition of rennet or calcium chloride. Furthermore, clots can be produced by the same method from metacaseinogen which has been reconverted into caseinogen by treatment with alkalis (with the usual precautions), provided that the former has not been changed too much by very prolonged action of water either at 37° or at higher temperatures. Where such additional changes have been brought about, there is evidence of the partial scission of phosphoric acid from the caseinogen molecule. In no case was it found possible to reconvert a metacaseinogen into a caseinogen with quite as high a solubility in ½ sat. $Ca(OH)_2$ as "natural" caseinogen. Alkali appears, therefore, to exert some slight subsidiary action.

* *Cf.* Brailsford Robertson.

The Chemical Nature of the Clot.

As already stated, clots, whether produced by calcium chloride alone, or by rennet alone, can be distinguished from metacaseinogen or caseinogen precipitates by the fact that, when moist, they yield an indiarubber-like mass on treatment with alcohol, whereas the unclotted material undergoes no visible change.

A systematic examination was made of the clots prepared by various processes with the object of determining their relationship to caseinogen and metacaseinogen. The rennet clot was formed by adding 1 c.c. of a commercial rennet solution to 50 c.c. of a saturated solution of "natural" caseinogen in $\frac{1}{2}$ sat. $Ca(OH)_2$. The mixture was made in a high cylinder in the cold, and was then placed in an incubator. After about 10 minutes, the whole had set to a solid clot, which was broken up, filtered off from the liquid, washed with ice-cold water, alcohol, and ether, and then air-dried. It was then boiled with absolute alcohol for about 10 minutes to destroy the ferment, and analysed. A portion was then dissolved in weak sodium hydroxide solution and reprecipitated, after filtration through pulp, by acetic acid, rapidly washed with ice-cold water after precipitation, and freed from water in the usual way.

The clot produced by calcium chloride was prepared by mixing the calcium caseinogenate solution with an equal volume of N/25 calcium chloride solution, and incubating the mixture until the clot had formed. This was then filtered off, washed with 50-per-cent. alcohol until the washings were free from chlorine, then with absolute alcohol and ether, and then air-dried. To free it from calcium it was treated in the same way as the rennet clot, *i.e.* redissolved in alkali (NaOH), and reprecipitated with the usual precautions.

It was found that the clots produced by rennet alone and by calcium chloride alone differed in one important particular, for whereas the calcium chloride clot (purified by solution in alkali and reprecipitation) gave with $\frac{1}{2}$ sat. $Ca(OH)_2$ a milky solution of high solubility (more than 30), which clotted on addition both of rennet and calcium chloride, the purified substance from the rennet clot yielded with the lime water of the same concentration only an opalescent solution (solubility 12·5), which gave a precipitate with an equal volume of N/25 calcium chloride solution, and no clot on addition of rennet.

The rennet clot differed therefore both from metacaseinogen and caseinogen, in that, even after re-solution in alkali, it no longer gave rise to a product capable of giving with $\frac{1}{2}$ sat. $Ca(OH)_2$ an opaque highly concentrated solution, from which clots can be formed.

Experiments were next carried out to ascertain whether the treatment with alcohol had caused the difference between the calcium chloride and the rennet clots. For this purpose a calcium chloride clot was obtained in the form of a dry powder, by the method described above, and then boiled for 10 minutes with alcohol, and purified by solution in alkali and reprecipitated. The preparation thus obtained also gave with $\frac{1}{2}$ sat. $Ca(OH)_2$ an opaque solution of high concentration (29·7) which did not clot on addition of rennet, and gave only an incomplete clot on addition of calcium chloride. Alcohol, therefore, alters both the calcium chloride and the rennet clots. The fact may be recalled that caseinogen on heating with alcohol is not altered, but yields a solution in $\frac{1}{2}$ sat. $Ca(OH)_2$, which readily clots both on addition of rennet and calcium chloride. The clot produced by calcium chloride alone, however, on re-solution in alkali, is readily converted into caseinogen, whereas the clot produced by rennet alone, even after redissolving in alkali, yields a product of low solubility in $\frac{1}{2}$ sat. $Ca(OH)_2$ from which no clot could be produced. It was unfortunately necessary to heat the rennet clot with alcohol to destroy the ferment, and as there is evidence that the calcium chloride clot is also altered by this treatment, the question as to whether the rennet clot can be reconverted into a clottable caseinogen remains for the present unsolved.

Numerous other experiments were carried out with the object of preparing a clottable calcium caseinogenate solution from the rennet clot. The ferment in one experiment was destroyed by heating with water, and then after drying with alcohol and ether was redissolved in sodium hydroxide solution and reprecipitated by acid. In another experiment the clot, after washing with ice-cold water, was directly dissolved (whilst still moist) in sodium hydroxide solution, reprecipitated, dried in the usual way, and then heated for a few minutes with boiling alcohol, a treatment which causes no change in caseinogen. In no case was a product of greater solubility than 17 to 20 in $\frac{1}{2}$ sat. $Ca(OH)_2$ obtained, which is only about half that of natural caseinogen. The solutions were in all cases translucent and yielded no clot either with calcium chloride or with rennet. The evidence obtained so far tends to indicate that rennet alters caseinogen in such a way that it is not reconvertible into caseinogen.

The fact that both descriptions of clot are altered by alcohol, whereas caseinogen is not, indicates that the latter undergoes some change during the process of clot formation. The fact, however, that the rennet clot, even after re-solution in alkali, gives a product of low solubility in lime water, whereas the calcium chloride clot gives one of high solubility (even after treatment with hot alcohol), indicates that the caseinogen under the influence of rennet

is converted into metacaseinogen, which undergoes some further alteration, whereas in the absence of rennet it can form a clot without undergoing this change. The clot produced by the direct action of rennet on milk, which was prepared in the same way as the rennet clot from pure calcium caseinogenate solution, behaved in the same way as the latter, yielding after purification a product of low solubility in lime water. It contained, as the analyses* indicate, products other than those derived from caseinogen, carried down from the milk. The analyses show furthermore that the nitrogen–phosphorus ratios in caseinogen, metacaseinogen (unless heated for too long a period), and in the clots produced both by calcium salts and rennet are the same. There is no evidence, therefore, of any proteoclastic digestion produced by the rennet. The change of caseinogen into metacaseinogen is not an essential for clot formation, which can, furthermore, be inhibited by the presence of various adsorbable substances. Owing to the lability of caseinogen, especially under the action of rennet, it is not possible to conclude from the experiments on milk-clot formation that the ferment exerts a direct antagonising influence on the substances inhibiting aggregation, although such an action is not by any means improbable. Nor is it possible from the above experiments to directly formulate the chemical changes which take place in clot formation. The evidence points to the fact that it is the free caseinogen which is changed. This substance, as a complex polybasic acid, can conceivably undergo many changes by the simple scission of the elements of water, and although it is not possible to express in the form of an equation the conversion of caseinogen into casein, such a change does not appear to be at all mysterious when considered from a chemical standpoint.

Summary and Conclusions.

1. A preliminary account is given of the action of calcium salts on sodium cholate (cholalate). When solutions of these substances are mixed, a clot is formed on heating. Investigations were carried out with the object of determining the relationships between the clotting time and the amounts and characters of the calcium salts. It was found that, in the case of those salts which raise the surface tension of water, the greater the concentration of the salt the shorter was the time required for clot formation. In the case of salts which lower the surface tension, on the other hand, increase of concentration decreased the clotting time only up to a certain limit of optimal concentration. Above this limit the clotting time was diminished, or the clot formation inhibited entirely. The more a salt

* These analyses are not published in this communication.

lowers the surface tension of water the narrower the limits of concentration within which clot formation is impossible. The inhibition of intravascular clotting after peptone injection is probably a similar phenomenon.

2. This inhibition of clotting is probably due to the adsorption of simple molecules by the more complex colloidal substances, which are thereby inhibited from aggregation to form a clot. The results suggested that in other cases, such as that of milk, the materials necessary for clot formation pre-exist, but that aggregation is prevented by the adsorption of simpler molecules from the system. The conception was formed that a ferment, for which the colloidal substances could act as a substrate, could clear the surface of such substances of adsorbed bodies and thus allow aggregation (clot) formation to take place. If such an action of ferments takes place it might be possible to explain the function of the intracellular ferments. If they act in the manner suggested, an aggregation equilibrium in the system—colloids (proteins, etc.), simpler adsorbable substances (extractives, etc.), ferment—would be maintained and would be probably necessary for the maintenance of the normal functions of the cell. There would, in this respect, exist a contrast between the "solid" tissues and the fluids of the body.

3. In attempting to apply this hypothesis to explain the clotting of milk, efforts were made to obtain a "natural" caseinogen. It is already known that caseinogen forms with alkalis solutions of very acid salts, and considerable differences were found in the individual preparations with regard to the amount of caseinogen dissolved by alkalis. The solubility in half-saturated lime water was employed as the criterion for differentiating the various preparations. It was found that if caseinogen is prepared in such a way that it is allowed to remain for as short a time as possible with acetic acid used for its precipitation (1 in 1000), a product is obtained which gives an opaque milky fluid containing nearly 8 per cent. of caseinogen. If such a preparation is heated with water at 37°, or allowed to stand with the acetic acid (1 in 1000) at room temperature, it gives rise to a product, the solubility of which in lime water is only about $\frac{1}{3}$ of that of natural caseinogen. This has been designated "metacaseinogen," the solution of which in half-saturated lime water is opalescent and not opaque. Metacaseinogen can be reconverted into caseinogen by solution in sodium hydroxide and precipitation with acetic acid, provided that the precautions are taken that the precipitate does not remain too long in contact with the acid. The solvent capacity of sodium hydroxide approximates to that of an equimolar (not equinormal) solution of calcium hydroxide.

4. The action of calcium chloride solutions on a saturated solution of

caseinogen in sodium hydroxide equimolar with half-saturated lime water was investigated. It was found that a precipitate was formed (apparently by double decomposition) only when the concentration of the calcium salt was within certain definite limits. The reactions form an "irregular series" similar to many others where one of the reacting substances is a complex colloid. If rennet is added to a mixture in which precipitation is inhibited by excess of calcium salt an action takes place, and a precipitate is formed shortly after the addition of the ferment.

5. If the optimal amount of calcium salt is present, precipitate formation can also be inhibited by the presence of milk serum, Witte's peptone, or even glycine. The addition of rennet to such mixtures can cause precipitation, provided that not too much inhibitory substance is present. The amount of calcium salt also influences the reaction, which depends therefore on the relative quantities of various products present in the system. The relative influence of these substances has not yet been investigated in detail. The precipitating power of calcium salts other than the chloride has also been investigated.

6. If solutions of calcium chloride or the salts of another alkaline earth are added to a saturated solution of natural caseinogen in $\frac{1}{2}$ sat. $Ca(OH)_2$ no precipitate is formed at room temperature. If, however, the mixtures are warmed slightly (to 25°), a typical clot is formed within certain limits of concentration of the calcium chloride. This shrinks, and gives on treatment with alcohol an indiarubber-like mass, and behaves, generally, in a manner characteristic of the milk clot obtained by rennet. In optimal concentrations of the calcium salt, clot formation can also be inhibited by the presence of milk serum, Witte's peptone and glycine. The addition of rennet to mixtures containing these inhibitory substances can cause the clot to form directly.

7. There is reason to believe that the clot is formed from free caseinogen or metacaseinogen and not from the calcium salt. The chief are :—

(*a*) The clot after addition of calcium chloride forms only on warming, and there is evidence that the calcium caseinogenate, under these conditions, undergoes hydrolytic dissociation.

(*b*) The clot can, however, form in the cold, if the caseinogenate solution is previously treated with carbon dioxide.

(*c*) The clot can be formed from sodium caseinogenate solutions, in the absence of calcium, if the latter are treated first with carbon dioxide and then with rennet.

8. Calcium (but not sodium) caseinogenate solutions clot on addition of rennet in the absence of calcium chloride. Clot formation under these conditions is, however, inhibited by relatively low concentrations of milk serum.

9. Solutions of calcium metacaseinogenate are, under optimal conditions of concentration, only incompletely precipitated by calcium salts, and do not clot on addition of rennet.

10. There is evidence that the clot formed on addition of rennet alone is formed from metacaseinogen, as it has a low solubility in half saturated lime water, whereas that formed by addition of calcium chloride alone is formed from caseinogen. Rennet appears also to cause some further change, as up to the present all attempts to reconvert the clot into natural caseinogen (by action of alkalis, etc.) have failed. In this respect, the casein differs from metacaseinogen. Clottable caseinogenate solutions can, however, be readily prepared from clots produced by the action of calcium chloride alone.

11. It is not possible to formulate accurately the relationships of caseinogen, metacaseinogen, and the clots to one another. Analyses negative the suggestion of anything of the nature of proteoclastic digestion on addition of the rennet. The products are possibly formed from one another by the scission or addition of the elements of water from or to the acid hydroxyl groups, and possibly the various products bear the same relation to one another as do, *e.g.* the pyro-, ortho-, and metaphosphates.

12. Although many of the facts appear to support the hypothesis as to ferment actions given above (para. 2), the same cannot be said to be definitely proved by the facts elicited in the study of the phenomenon of milk clotting. The process is rendered more difficult of comprehension by the peculiar instability of caseinogen. There is no doubt, however, that in milk the clot formation depends upon the presence of four series of substances in the system, viz., simple inhibitory substances, colloids, ferment and calcium salt, even if their relative actions cannot be formulated in as simple a manner as that suggested.