CLXXXI. THE HEAT-COAGULATION OF CASEINOGEN.

I. THE RÔLE OF PHOSPHORUS CLEAVAGE.

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INVESTIGATIONS into the heat-coagulation of proteins, notably of egg-albumin and oxyhaemoglobin, indicate that two separate reactions are involved, denaturation and flocculation. Denaturation, which precedes flocculation, involves an irreversible change in the structure of the molecule, probably of a hydrolytic nature. Flocculation is a purely physical phenomenon, being largely dependent on the presence of electrolytes and, under suitable conditions, reversible. Chick and Martin [1912] and Lewis [1926], the former studying egg-albumin and the latter oxyhaemoglobin, have shown that the denaturation of these proteins, as measured by the amount of coagulation in the presence of small quantities of electrolytes, is a unimolecular reaction whose constant k can be accurately determined provided that the p_H of the solution is kept constant throughout the course of the reaction.

Caseinogen is usually regarded as a relatively heat-stable protein, but it is occasionally found that drastic heat treatment (such as occurs in the sterilisation of evaporated milk) will lead to its complete coagulation. Investigations into the causes of such coagulation have in the past been chiefly concerned with the influence of contributory factors, such as the concentration of electrolytes and the presence of protective colloids (*e.g.* lactalbumin) on the heat-stability of the caseinogen. No attempt appears to have been made to study the effect of drastic heat treatment on the protein itself. Yet it is apparent from the results of a number of investigations that the caseinogen molecule is easily disrupted by the action of various agents; more especially is this true of the cleavage of phosphorus from the caseinogen molecule. Thus Rimington and Kay [1926] and Stirling and Wishart [1932] have shown that trypsin-kinase liberates the phosphorus from caseinogen with comparative ease; in two experiments carried out by the latter authors liberation of the entire phosphorus occurred in less than one hour. Rimington and Kay [1926] have also shown that mild alkaline hydrolysis at 37° results in complete dephosphorisation of caseinogen.

It was not therefore surprising to find that, in preliminary experiments in which neutral solutions of sodium caseinogenate were heated in closed tubes at 120° , there was a definite cleavage of phosphorus. It was felt that these preliminary experiments warranted more detailed study. The results of this study are described in the present paper.

I. THE EFFECT OF HEAT ON THE CLEAVAGE OF PHOSPHORUS FROM CASEINOGEN.

Technique.

Preparation of solutions. Caseinogen "nach Hammarsten" was used throughout. It contained 9 % moisture, the nitrogen and phosphorus contents (calculated on dry matter) being 15.08 and 0.83 % respectively. Solutions were prepared as follows:

3.5 % sodium caseinogenate. 3.5 g. caseinogen were mixed with sufficient water and 0.1 *N* NaOH to give, when diluted to 100 ml., a final concentration of 0.0175 *N* NaOH.

3.5 % calcium caseinogenate. 3.5 g. caseinogen were mixed with sufficient water and solid Ca(OH)₂ to give, when diluted to 100 ml., a final concentration of 0.0175 *N* Ca(OH)₂.

The solutions were mechanically stirred until the caseinogen was completely dissolved. The final solutions appeared homogeneous, the solutions of sodium caseinogenate being almost clear and those of calcium caseinogenate opalescent but transparent in thin layers. The p_H of the solutions lay between 6.5 and 7.0. It may be noted here that, immediately they were immersed in the glycerol-bath at 120°, both solutions became very turbid. There was, however, no flocculation, and the solutions, if removed from the bath, remained stable for an indefinite period.

Heating control. The solutions were heated in an open tinned copper bath containing glycerol. The bath was fitted with an efficient stirrer, and the temperature was controlled by a thermionic valve relay in conjunction with a contact thermometer (Baily, Grundy and Barrett). The temperature used throughout the entire series of experiments was 120° and did not vary by more than 0.25°.

As the experiments were carried out above boiling-point it was necessary to use closed tubes for the caseinogen solutions. A very cheap and efficient method was devised. Small heavy-walled pyrex tubes were used, each being closed with a rubber stopper. Each tube was fixed tightly in a metal clamp, a small piece of rubber being inserted as a cushion between the bottom of the tube and the clamp (see Fig. 1). This arrangement enabled the observer to shake, invert and remove each tube independently. After removal from the bath the tubes were cooled quickly in running water.

Analysis. Nitrogen determinations were made by the micro-Kjeldahl method. Phosphorus was estimated by the method of Fiske and Subbarow [1925].

Experimental procedure. A number of tubes, each containing 5 ml. of 3.5 % sodium (or calcium) caseinogenate, were immersed in the glycerol-bath at 120° for periods of from 1 to 5 hours. The tubes were removed from the bath at appropriate intervals, cooled and, where necessary, shaken to break up any coagulum which had been formed. 5 ml. of 10 % trichloroacetic acid were then added to each tube. After standing for 5 minutes the contents were filtered through a No. 1 Whatman filter. Nitrogen and phosphorus determinations were made on aliquot portions of the filtrate. Each experiment was carried through in duplicate.

Fig. 1.

Discussion of results.

Typical curves showing the rate of liberation of acid-soluble phosphorus and nitrogen from a 3.5 % solution of sodium caseinogenate are given in Fig. 2. The equivalent values for a 3.5 % solution of calcium caseinogenate are given in Fig. 3.

Fig. 2. Effect of heat on 3.5 % solution of sodium caseinogenate.

Fig. 3. Effect of heat on 3.5 % solution of calcium caseinogenate.

It will be seen that the general trend of the results is the same for both the sodium and calcium salts, there being a rapid liberation of acid-soluble phosphorus and a relatively slow liberation of acid-soluble nitrogen. Moreover, there appears to be no slowing off in the liberation of nitrogen when the entire phosphorus of the caseinogen has become acid-soluble. This result is comparable with that found by Rimington and Kay [1926] for mild alkaline hydrolysis but differs markedly from the findings of Stirling and Wishart [1932] in their study of the action of trypsin-kinase on caseinogen. The relatively small amount of nitrogen liberated by heat treatment, and the fact that (as shown in Figs. 2 and 3) nitrogen cleavage takes place at the same rate with both the sodium and calcium

salts, while the rate of phosphorus cleavage differs for these two salts, provide strong evidence that the effect of heating is to liberate phosphorus as inorganic phosphate and not in organic combination.

Rimington and Kay found that alkaline hydrolysis of caseinogen resulted in the production of acid-soluble nitrogen in the form of ammonia. In the present experiments no estimations of free ammonia were made, but the fact that the solutions tended to become more acid as heating proceeded precludes the formation of any considerable quantity of ammonia. It appears from these facts that the mode of liberation of nitrogen from the caseinogen molecule by heat treatment differs from that resulting from treatment with alkali.

As regards the rate of liberation of acid-soluble phosphorus, conditions were not sufficiently constant to allow for any final conclusion as to the order of the reaction, since there was invariably an increase in the acidity of the solution during heating. There is, however, one marked difference between the curves for the sodium and calcium salts. It will be seen that with the sodium salt, 100 % of the phosphorus is rendered acid-soluble within 5 hours, whereas with the calcium salt only 80 % is rendered acid-soluble. It appeared at first sight that this might be due to the fact that with the calcium salt a clot was formed within 2 hours, and that in spite of vigorous shaking part of the phosphorus might remain embedded in this clot. Such an explanation would not, however, account for the difference in the amounts of phosphorus which were rendered acid-soluble within the first hour (*i.e.* 54 and 37 % respectively) during which no coagulation occurred. Moreover, the form of the curve for the calcium salt indicates that the reaction is practically complete when only 80 % of the phosphorus is in the acid-soluble form. It seems possible that the explanation of this marked difference between the sodium and calcium salts lies in the catalytic effect of Na ions.

II. THE PREPARATION AND PROPERTIES OF HEAT-DEPHOSPHORISED CASEINOGEN.

Preparation of heat-dephosphorised caseinogen.

One litre of 3.5 % sodium caseinogenate (p_H 6.7) was prepared as previously described and placed in a 2 litre flask. The mouth of the flask was covered with a thin sheet of cellophane and the flask heated in an autoclave. In about 30 minutes the pressure had reached 1 atmosphere (121°) and it was retained at this value for 4 hours. A further 30 minutes was allowed for the pressure to fall, and the flask was then removed and cooled in running water. No coagulation occurred during the heating, but a thin skin was formed on the bottom of the flask. The heated solution was definitely pink in colour. The solution was then treated with small quantities of 33 % acetic acid until maximum precipitation took place [Rimington and Kay, 1926]. The gelatinous precipitate was filtered off through linen, well washed with water and redissolved in dilute NaOH. It was then reprecipitated with 33 % acetic acid. At this stage the precipitate appeared white, but it soon darkened to brown even though not directly exposed to air. After settling, decanting off the supernatant liquid and thorough washing, the precipitate was again filtered through linen and well drained. It was next transferred to a mortar and ground with absolute alcohol for about an hour. On first contact with alcohol the precipitate became a gummy mass, but with continued grinding it became a friable powder. This was filtered off on a Büchner funnel, re-extracted with fresh alcohol for another hour, again filtered off and finally allowed to dry in a current of air. The final product was

a brownish pink powder. The moisture content (mostly alcohol) was 15 %. This could be reduced by treatment with ether to about 10 %, but the product so obtained was less easily soluble in alkali; maximum yield, 49 %.

Properties of heat-dephosphorised caseinogen.

In order to compare the properties of the heat-dephosphorised caseinogen with those of the untreated caseinogen and of Rimington and Kay's alkali-dephosphorised caseinogen, samples of the latter two products were also prepared. The alkali-dephosphorised caseinogen was prepared according to Rimington and Kay's directions. The control sample of caseinogen was prepared from an untreated solution of 3.5 % sodium caseinogenate, the methods of precipitation and purification being identical with those used for the dephosphorised products. The nitrogen and phosphorus contents of the three preparations (expressed as % of dry matter) were as follows:

	N (%)	P (%)
Original (untreated) caseinogen	15.08	0.830
Heat-dephosphorised caseinogen	13.86	0.032
Alkali-dephosphorised caseinogen	13.56	0.016

Two methods were used for differentiating the three products, (a) the determination of their titration curves, and (b) the determination of their relative rates of coagulation when heated at 120°.

(a) *Titration curves.*

Technique. 1 % solutions (on dry matter basis) of each product were prepared. For acid-binding capacity the solutions were made up with standard HCl (approximately 0.1 *N*) of sufficient amount to give an initial p_H of 2.0–3.0; for base-binding capacity sufficient standard NaOH (approximately 0.1 *N*) was used to give an initial p_H of 8.0–9.0. The amount of acid or alkali added was noted. The method of determining the combining capacity was that of "back-titration" from the initial p_H to the region of the isoelectric point. For this purpose known amounts of standard alkali or acid were added to aliquot portions of the original solutions, and the p_H was determined after each addition.

For base-binding capacity the p_H measurements were made with a glass electrode. For acid-binding capacity a quinhydrone electrode was used in combination with a saturated calomel half-cell. With both methods of determination the accuracy of the measurements was within 0.5 millivolt.

The method of calculation of the equivalents of acid or base bound was as follows:

Acid-binding. At any given p_H , equivalents bound per g. protein = $[H]$ added – $[H]$ found, where

$[H]$ = concentration of acid in equivalents.

The concentration of acid added was obtained directly from the amount of HCl added *minus* the amount of NaOH added (expressed in equivalents). The concentration of free acid ($[H]$ found) was calculated from the observed p_H .

Base-binding. At any given p_H , equivalents bound per g. protein = $[OH]$ added – $[OH]$ found, where

$[OH]$ = concentration of alkali in equivalents.

The concentration of alkali added was obtained directly from the amount of NaOH added *minus* the amount of HCl added (expressed in equivalents). The concentration of free alkali ($[OH]$ found) was calculated from the observed p_H .

The ionic product for water was taken as $K_w = 0.74 \times 10^{-14}$ at 18° [Michaelis, 1926]. The degree of dissociation, α , was taken as unity throughout the concentrations employed. Hoffmann and Gortner [1925] have pointed out that the degree of dissociation as determined by conductivity methods does not agree with the values obtained by potentiometric methods and suggest the use of the latter in calculating combining capacities. It was found that, up to the concentrations used in the present experiments, this correction was not of sufficient magnitude to be considered necessary.

Fig. 4. Titration curves of *A*, untreated caseinogen; *B*, alkali-dephosphorised caseinogen; *C*, heat-dephosphorised caseinogen.

Results. The titration curves of the three products are shown in Figs. 4 and 5. The curves for the untreated caseinogen coincide fairly closely with those recorded by Pertzoff and Carpenter [1932]. It will be seen, however, that both alkaline hydrolysis and heat treatment have effected marked alterations in the base-binding capacity and, to a less extent, in the acid-binding capacity. Moreover, it is of special significance that the curves for the two dephosphorised products differ markedly, the alkali-dephosphorised caseinogen having a higher and the heat-dephosphorised caseinogen a lower base-binding capacity than untreated caseinogen. It has already been noted that the mode of liberation of phosphorus by heat appears to be identical with that caused by alkaline hydrolysis. On the other hand, it has been shown that the mode of cleavage of nitrogen probably differs in the two treatments. It seems likely therefore that the differences in combining capacity are associated with the cleavage of nitrogen rather than of phosphorus.

It should be added that, so far as the two dephosphorised products are concerned, it is not legitimate to consider these as specific proteins of fixed chemical

composition, as in the case of caseinogen. Owing to variations in the rate of heating and cooling of the autoclave, as well as to other conditions which cannot be kept exactly constant, two successive samples of the heat-dephosphorised caseinogen may vary somewhat in composition. The same criticism holds for the alkali-dephosphorised caseinogen. Such variations in composition are, however, unlikely to affect the acid- and base-binding capacities to any serious extent, and it is reasonable to assume that the curves shown in Figs. 4 and 5 represent typical acid- and base-binding capacities of the two types of dephosphorised product.

Fig. 5. Titration curves of A, untreated caseinogen; B, alkali-dephosphorised caseinogen; C, heat-dephosphorised caseinogen.

(b) *The rate of heat coagulation.*

Technique. 3 % solutions of the calcium salts of each of the three products already described were prepared as noted under (1) and (2) below. 5 ml. portions of the solutions were transferred to stoppered tubes, and the tubes were heated in a glycerol-bath at 120° (as previously described) for periods of from 1 to 5 hours. After removal from the bath the tubes were immediately cooled in water, the coagulated material was separated by centrifuging at 3000 r.p.m. for 5 minutes, and aliquot portions of the supernatant liquid were removed for analysis. Determinations were made of the total and non-protein nitrogen, the percentage of uncoagulated protein in the supernatant liquid being calculated by difference.

1. *Solutions containing minimum quantities of $\text{Ca}(\text{OH})_2$.* The first series of experiments was carried out using the minimum quantities of $\text{Ca}(\text{OH})_2$ necessary to give approximately neutral solutions. For this purpose the requisite amounts

of alkali were calculated from the titration curves already discussed (Fig. 5). The actual quantities of $\text{Ca}(\text{OH})_2$ used, per 100 ml. of 3 % solution, were 0.061 g. for the untreated caseinogen; 0.051 g. for the heat-dephosphorised caseinogen; and 0.093 g. for the alkali-dephosphorised caseinogen. The p_{H} values of the solutions varied from 6.83 to 6.99. The relative rates of coagulation of these solutions are shown (as unbroken lines) in Figs. 6, 7 and 8.

Fig. 6. Untreated caseinogen.

Fig. 7. Heat-dephosphorised caseinogen.

Fig. 8. Alkali-dephosphorised caseinogen.

It will be seen that the dephosphorised products coagulate very much more rapidly than the untreated caseinogen, and that the form of the coagulation curve obtained with the dephosphorised products differs fundamentally from that obtained with the caseinogen. Thus there is, during the first hour of heating, a rapid coagulation of the former products, over 70 % of the heat-dephosphorised caseinogen and nearly 60 % of the alkali-dephosphorised caseinogen being coagulated within this period. Within the same period barely 10 % of the untreated caseinogen is coagulated. After nearly 3 hours' heating, however, there is a sudden and marked increase in the rate of coagulation of the untreated caseinogen. If reference is made to Fig. 3, it will be seen that this increase coincides with the liberation of from 60 to 70 % of the phosphorus, so that, in that part of the curve where coagulation is most rapid, we are, in fact, dealing with a partly dephosphorised product, and not with untreated caseinogen.

The fact that the two dephosphorised products give coagulation curves of similar type, and that maximum coagulation occurs in the untreated caseinogen only after it has been partly dephosphorised, demonstrates the enhanced heat-

sensitivity which is associated with loss of phosphorus from the caseinogen molecule.

2. *Solutions containing excess Ca ions.* If reference is made to Fig. 5 it will be seen that the base-binding capacity of untreated caseinogen is higher than that of the heat-dephosphorised caseinogen. At p_H 6.9 this difference amounts to nearly 10×10^{-5} g.-eq. of alkali per 1 g. of protein. That is to say, the production of heat-dephosphorised caseinogen from a neutral solution of caseinogen will decrease the base-binding capacity by 16 %. It seemed reasonable to assume that this reduction in base-binding capacity would be accompanied (with the calcium salt, for example) by an actual liberation of calcium, either as free ions or in association with inorganic phosphate or protein degradation products. Now it has long been recognised that the presence of calcium ions tends to reduce the heat-stability of solutions of caseinogen, and it appeared possible, therefore, that such a liberation of calcium might be an important factor in accelerating coagulation of the heated caseinogen and/or of its dephosphorised product.

In order to investigate the effect of excess Ca ions on the rate of heat-coagulation of heat-dephosphorised caseinogen, a solution of the dephosphorised product was prepared which contained the same quantity of Ca(OH)_2 as was employed in making up the neutral solution of the untreated caseinogen (*i.e.* 0.061 g. Ca(OH)_2 per 100 ml. in place of 0.051 g. Ca(OH)_2). The p_H of the solution was readjusted to neutral point by adding small quantities of 0.1 *N* HCl. The resultant solution can be considered as a solution of Ca caseinogenate in which the entire protein has been dephosphorised, but in which the original amount of calcium is still present. A similar additional quantity of Ca(OH)_2 was added to a solution of untreated Ca caseinogenate (and the p_H adjusted with HCl) in order to investigate the effect of the excess Ca ions on the heat-coagulation of the unaltered caseinogen.

The relative rates of coagulation of these two solutions are shown in Figs. 6 and 7. It will be seen that the presence of excess Ca ions accelerates the coagulation of both solutions, the effect being more striking with the untreated caseinogen than with the dephosphorised product. The rate of coagulation of the untreated caseinogen, however, still lags behind that of the dephosphorised product throughout the greater part of the curve, the relative proportions coagulated at the end of one hour being 30 and 75 % respectively. Moreover, as has been pointed out in the previous section, the greater part of the caseinogen will have been dephosphorised within the first 2–3 hours of heating, so that in the latter half of the curve we are really dealing with the dephosphorised product and not with untreated caseinogen.

DISCUSSION AND SUMMARY.

It is now possible to summarise in general terms the changes which result in, and accompany, the heat-coagulation of caseinogen solutions. If, for example, a neutral solution of calcium caseinogenate is heated at 120°, the following reactions will occur:

1. There will be a fairly rapid liberation of phosphorus from the caseinogen molecule, with the consequent conversion of the greater part of the caseinogen into a dephosphorised product. Concurrently a small part of the protein will be degraded, as shown by a progressive increase in the non-protein nitrogen fraction.

2. The dephosphorised product, being less heat-stable than the original caseinogen, will be rapidly coagulated by the continued heat treatment.

3. Since the base-binding capacity of the dephosphorised product is lower than that of caseinogen, its dephosphorisation will be accompanied by a liberation of calcium, either in ionic form or in association with inorganic phosphate or protein degradation products.

4. The excess Ca ions thus liberated will increase the heat-sensitivity of both the original caseinogen and the dephosphorised product, with consequent acceleration in the rate of coagulation.

It should be made clear that, while the two main reactions noted above, *i.e.* liberation of phosphorus and concurrent liberation of calcium, appear to play a large part in the heat-coagulation of solutions of calcium caseinogenate, they are probably not the only factors involved. For instance, changes in p_H of the solution and alterations in the degree of dispersion and the degree of hydration of the protein particles will undoubtedly exert a considerable influence on the rate of heat-coagulation. Moreover, it must be borne in mind that the reactions described in this paper have been induced by abnormally drastic heat treatment, *i.e.* a temperature of 120° and heating for periods up to 5 hours. The results cannot, therefore, be directly applied to the type of heat coagulation which takes place in the manufacture of evaporated milks.

Nevertheless the results are of importance in that they provide an example of denaturation in which a distinct structural change, namely dephosphorisation of the molecule, forms a preliminary to coagulation. Such a finding appears to be without parallel in previous studies of denaturation and heat coagulation.

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REFERENCES.

- Chick and Martin (1912). *J. Physiol.* **43**, 1; **45**, 261.
Fiske and Subbarrow (1925). *J. Biol. Chem.* **66**, 375.
Hoffman and Gortner (1925). Colloid Symposium Monograph, **2**, 209.
Lewis (1926). *Biochem. J.* **20**, 965.
Michaelis (1926). Hydrogen ion concentration, **1**, 25 (English trans.).
Pertsoff and Carpenter (1932). *J. Gen. Physiol.* **16**, 257.
Rimington and Kay (1926). *Biochem. J.* **20**, 777.
Stirling and Wishart (1932). *Biochem. J.* **26**, 1989.