STUDIES ON THE ACTION OF RENNET ON CASEIN AND THE NATURE OF CLOTTING

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Summary

Casein and paracasein preparations have been compared by various methods, including titration techniques, solubility, and ultraviolet spectra of the proteins and their azo-derivatives. It is suggested that rennet may release imidazole or phenolic groups from intramolecular linkage. The liberation of proteoses was confirmed but their presence does not appear to be essential for clotting. Support is given to the view that α-casein is more affected by rennet than are other casein components. The stability of the clots and the effect of electrolytes on the clotting process have been examined.

I. Introduction

The mechanism of milk clotting can be resolved into two stages: the conversion of casein into paracasein under the influence of the rennet, and the coagulation of the paracasein in the presence of calcium ions. Linderstrøm-Lang (1929), who first demonstrated the inhomogeneity of casein, advanced the hypothesis that rennet destroys, or inactivates, a "protective colloid" component, thus rendering unstable the calcium caseinates corresponding to the other components, and leading to precipitation or clotting. Alexander (1912) had previously put forward a somewhat similar hypothesis, in which lactalbumin was considered as the protective colloid. The work of Cherbuliez and Meyer (1933), Cherbuliez and Jeannerat (1939), and Cherbuliez and Baudet (1950) has indicated that α-casein may be regarded as the protective colloid in Linderstrøm-Lang's theory and that the calcium salts of β- and γ-casein, which are themselves insoluble, are kept in solution by the soluble calcium salt of α-casein, with which they are associated. Nitschmann and Lehmann (1947) have provided further support for Linderstrøm-Lang's hypothesis by showing that calcium ions cannot differentiate between casein and paracasein particles in a mixed solution.

Hammarsten (1872) postulated that the action of rennet on casein resulted in an insoluble curd and a soluble whey-protein (usually known in the literature as "Hammarsten's proteose"). Cherbuliez and Baudet (1950) regarded their soluble δ-fraction as this proteose. Earlier Bosworth (1913) had claimed that the appearance of the proteose was not due to the action of the rennet and in view of some confusion in the literature clarification of the following points seemed desirable: whether the enzyme is really responsible for the liberation

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of the proteose, and, if this is so, whether the reaction or the proteose itself are essential for the clotting process.

The assumption sometimes made that the rupture of peptide bonds is the basic feature of the casein-paracasein transformation probably arises from the observation that various proteolytic enzymes can coagulate milk, but it has actually received little experimental support. Wright (1924) had concluded from the similarity of the racemization curves for casein and paracasein that rennet does not cause proteolytic cleavage of the protein, and Holter (1932) was able to obtain, without apparent increase in amino or carboxyl groups, paracasein which could be completely precipitated by calcium ions. More recently Nitschmann and Varin (1951) have concluded that clotting can take place with negligible proteolysis, amounting to the rupture of only one peptide link in 10,000. Berridge (1945) demonstrated that commercial rennet is a mixture of pepsin and rennin, and that rennin itself also exhibits considerable proteolytic activity. The relevant question therefore is not whether proteolysis of the casein occurs, but whether it is essential for coagulation. Nitschmann and Lehmann (1947) suggested that the essential chemical change in the transformation of casein into paracasein might not lie in a primary valence reaction, but that the rennet might act as a specific "denaturase" for one of the casein components.

Some clarification of these questions was sought in the experiments described below.

II. Materials and Methods

(*a*) *Protein Preparations*

Casein and α-casein for the experiment described in Section III(*e*) were prepared from the same batch of milk according to the methods of Warner (1944), except for minor deviations.

Casein and paracasein for comparative studies were prepared under strictly parallel conditions, except for the rennet treatment, as described by Higgins and Hayes (1951). Two pairs of preparations were used.

(*b*) *Acid Hydrolysates*

These were prepared by refluxing 0.5 g air-dry protein with 10 ml 6N hydrochloric acid for 20 hr.

(*c*) *Rennet*

A commercial preparation (New Zealand cheese rennet) was used.

(*d*) *Electrometric Titration*

The pH values were measured by means of a glass electrode system. The proteins (2.0 g) were dissolved in 40 ml N/20 sodium hydroxide and titrated with 1N hydrochloric acid. Acid groups were calculated from the equivalent between pH 6 and pH 2. Controls were run in the absence of protein and appropriate corrections applied.

(*e*) *Formol Titration*

The method of Kekwick and Cannan (1936) was adopted. The formaldehyde was neutralized, the protein solutions (1 g in 50 ml N/50 sodium hydroxide) were adjusted to pH 9, and were titrated back to this point after the addition of the formaldehyde.

(*f*) *Titration in Organic Solvents*

The methods of Linderstrøm-Lang and Jacobsen (1940) were applied, the titrations in ethanol being carried out with N/10 sodium hydroxide in 90 per cent. ethanol to the thymolphthalein end-point (normally pH 9.3-10.5), and those in acetone with N/10 hydrochloric acid in 90 per cent. ethanol to the naphthyl red end-point (normally pH 3.7-5.0). The original solutions were made up by dissolving 0.2 g protein, with agitation, in 20 ml N/20 sodium hydroxide and adjusting to pH 6, following Lea and Hannan (1950), with 1N hydrochloric acid (*c.* 0.8 ml). Titrations were carried out after the addition of (i) 15 ml ethanol plus 2.5 ml thymolphthalein (0.5 per cent.), (ii) 50 ml ethanol plus 2 ml thymolphthalein, (iii) 10 ml acetone plus 0.5 ml naphthyl red (0.01 per cent.), (iv) 30 ml acetone plus 1 ml naphthyl red. Controls were run on volumes of water equal to those of the protein solutions, adjusted to pH 6 and with the solvent and indicator added.

(*g*) *Total Nitrogen*

The Kjeldahl method was used, with parallel duplicates of each preparation.

(*h*) *Amino Nitrogen*

Determinations were made by the Van Slyke nitrous acid procedure in the manometric apparatus (Peters and Van Slyke 1932).

(*i*) *Optical Absorption*

Extinctions and absorption spectra were measured with a Unicam S.P. 500 spectrophotometer, using 1-cm cells and water as the reference medium. No transverse measurements of the scattered radiation were made, but high optical clarity was assumed in dilute protein solutions which had been filtered through sintered glass.

(*j*) *Solubility*

The proteins (0.05 g) were equilibrated at 25°C with 5 ml solvent and centrifuged to remove the undissolved protein. Extinctions were measured at 277 mμ on the supernatants.

(*k*) *Coupling with Diazotized Sulphanilic Acid*

Coupling was carried out as described previously (Higgins and Fraser 1952). The proteins (0.01 g) were first dissolved in *c.* 5 ml N/20 sodium hydroxide for 5 min and the solutions acidified with 1N hydrochloric acid.

III. Procedure and Results

(*a*) *Proteolysis. Acid and Basic Groups*

The various titration techniques were used to determine the state of the acid and basic groups in casein and paracasein preparations. As these procedures involved dissolving the protein in sodium hydroxide, an examination was made of the rate of hydrolysis of casein under the conditions used. The rate of rupture of peptide bonds was found, by titrations in alcoholic sodium hydroxide, to be about 2 per hour per 10^{5} g casein in the initial stages, which is not great enough to introduce serious errors during the periods used to dissolve the casein (usually about ½ hr).

The pH values obtained upon dissolving 1.0 g protein in 50 ml N/50 sodium hydroxide were, for paracasein: 8.34, 8.32, and for casein: 9.55, 9.40. After adjustment to pH 9 and addition of formaldehyde for the formol titration the pH values were respectively 6.47 and 6.44.

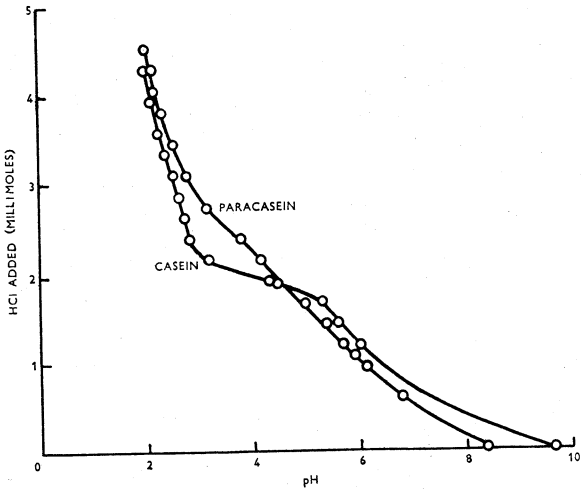

Fig. 1.—Electrometric titration of casein and paracasein. Amount of 1N HCl added to 2.0 g protein dissolved in 40 ml 0.05N NaOH.

Typical electrometric titration curves for casein and paracasein are shown in Figure 1. The titration equivalent between pH 2 and pH 6 has been used as a measure of free carboxyl groups by, for example, Herriott (1947), who drew attention to the uncertainty arising from the overlapping of the carboxyl and histidine titration regions. Although the method has been used for casein by Lea and Hannan (1950), a further objection arises from the presence of phosphoric acid groups. These have been taken into account by Hipp, Groves, and McMeekin (1952) on the assumption that the phosphoric acid affects the titration curve of casein as it does that of egg albumin (Cannan, Kibrick, and Palmer 1941). The total acid groups titratable between pH 2 and pH 6 have been calculated and are given in Table 1. The data have been corrected for

the controls, but as these are the same for each preparation, the magnitude of the increase upon rennet treatment is not affected.

TABLE 1

COMPARISON OF CASEIN AND PARACASEIN FROM TITRATION DATA

Titration (see text)	No. of Groups per 10^5 g Air-dried Protein			
	Casein	Paracasein	Increase	
Electrometric	131	152	21	
Formol	73	69	−4	
Base in ethanol (15 ml)	58·6	80·1	21·5	Mean 21
Base in ethanol (50 ml)	90·4	110·0	19·6	
Acid in acetone (10 ml)	77·6	92·4	14·8	Mean 15
Acid in acetone (30 ml)	88·8	103·0	14·2	

The ϵ-amino groups of lysine were calculated from the formol titration equivalents. The apparent small decrease upon rennet treatment (Table 1) may be due to the difference in ionic strength consequent upon adjusting to pH 9; Kekwick and Cannan (1936) drew attention to the large effect of salt upon the ionization of the protein.

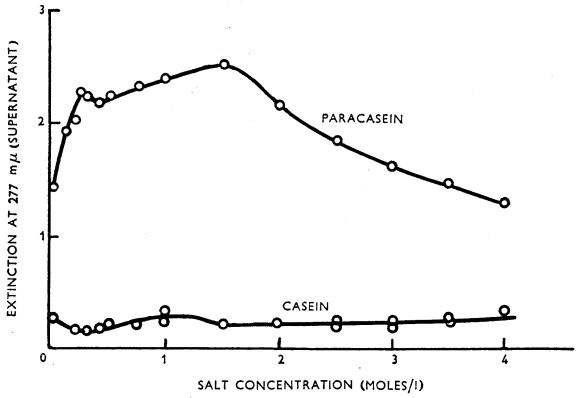

Fig. 2.—Solubility of casein and paracasein in the absence of calcium.

The interpretation of the ethanol and acetone titration data (Table 1) varies according to whether or not preliminary neutralization of the protein solution is effected (Richardson 1934), and there is also some disagreement between various authors (Richardson 1934; Linderstrøm-Lang and Jacobsen 1940; Lea and Hannan 1950).

The liberation of proteose by rennet was confirmed experimentally by examination, against appropriate controls, of the supernatants from the clotted

sodium caseinate solutions of concentration 3.1 per cent. and pH 6.7, after adjustment to pH 4.2-4.3. The tests, which are described in detail in an unpublished report by the authors, showed that the material liberated from the protein by rennet action was inhomogeneous and could be fractionated by conventional methods. It was observed that paracasein after removal of the proteose-containing supernatant and re-dissolving (3 per cent. solution) could still be clotted with calcium chloride.

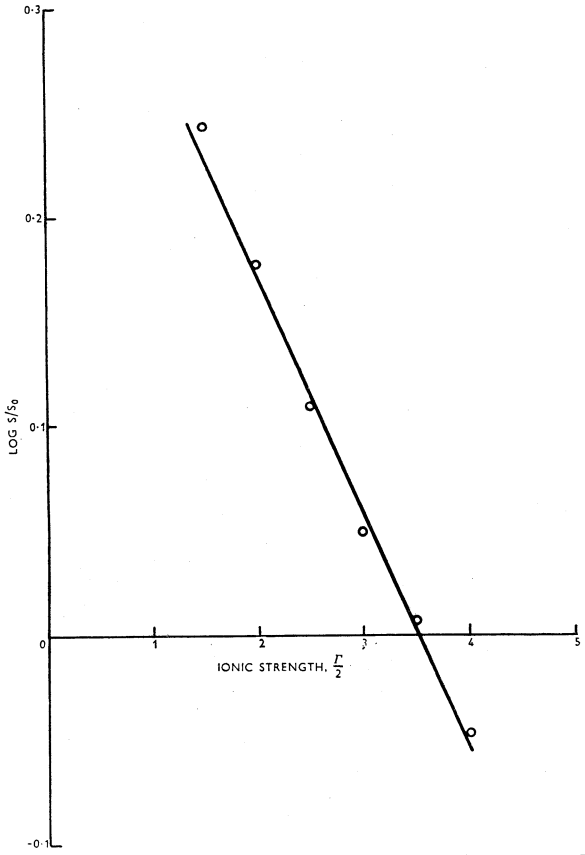

Fig. 3.—Salting-out of paracasein in ammonium chloride.

(*b*) *Solubility of Casein and Paracasein*

The solubility of casein in solutions of neutral salts is very considerably increased upon treatment with rennet, as shown in Figure 2 for ammonium chloride solutions. Apart from its greater solubility, paracasein exhibits very much greater sensitivity towards the concentration of salt. The subsidiary maximum at *c.* 0.25 mol/l suggests the presence of a component or group of components which can be salted out at concentrations much lower than that at which the main component reaches its maximum solubility (*c.* 1.5 mol/l). In Figure 3 the salting-out characteristics of paracasein are shown above this concentration. It is not even possible to derive such a curve from the data for

casein for which log S/S_0 is practically zero. From the slope of the line in Figure 3 the salting-out constant for paracasein can be calculated (Cohn and Edsall 1943) as 0.114.

Despite its greater solubility in salt solutions at protein concentrations not greatly in excess of the solubility, paracasein shows a lower solubility at high protein concentrations. Thus when 16 g protein were equilibrated with 100 ml M/15 phosphate buffer of pH 6.9 by vigorous shaking at 25°C, the apparent solubilities were:

Casein 11.1 g/100 ml;
Paracasein 3.6 g/100 ml.

TABLE 2
ULTRAVIOLET ABSORPTION OF CASEIN AND PARACASEIN

Protein	Solvent	Extinction (1 g/l)	
		277, 280 mμ	320 mμ
Casein (12·15% N*)	M/15 phosphate buffer pH 6·8	1·02 (277)	0·231
	M/20 borax buffer pH 9·2	0·98 (277)	0·222
	N/10 sodium hydroxide	1·07 (280)	0·214
Paracasein (12·23% N*)	M/15 phosphate buffer pH 6·8	1·06 (277)	0·240
	M/20 borax buffer pH 9·2	1·03 (277)	0·230
	N/10 sodium hydroxide	1·10 (280)	0·222

* On an air-dry basis. Correction for moisture would increase these figures considerably.

(*c*) *Ultraviolet Absorption of Casein and Paracasein*

Porcher, Volkringer, and Brigando (1934) demonstrated the similarity of the ultraviolet absorption spectra of casein and paracasein. Spectra observed on one pair of the present preparations in phosphate buffer of pH 6.9 showed a maximum at *c.* 277 mμ, but the extinction coefficient at this wavelength was nearly 4 per cent. higher for paracasein after correcting for haze by subtraction of the extinction at 320 mμ (Beaven and Holiday 1952). This effect was examined, in relation to total nitrogen and at different pH values, with casein and paracasein derived from another batch of milk. The extinctions for paracasein corrected for haze and for nitrogen were higher in each case than those for casein by amounts in the vicinity of 3 per cent., as shown in Table 2.

Both casein and paracasein solutions were found to conform closely to Beer's Law at 280 mμ up to concentrations of at least 1 g/l when dissolved in phosphate buffer of pH 6.8.

(*d*) *Reaction of Casein and Paracasein with Diazonium Compounds*

The results of previous studies on the reaction of amino acids with diazonium compounds have been applied to the problem of the casein-paracasein

transformation. The spectra shown in Figures 4 and 5 refer to different series of intact casein and paracasein preparations, made from two batches of milk. Similar experiments were made with acid hydrolysates prepared from the same protein preparations used in obtaining the spectra shown in Figure 4. The

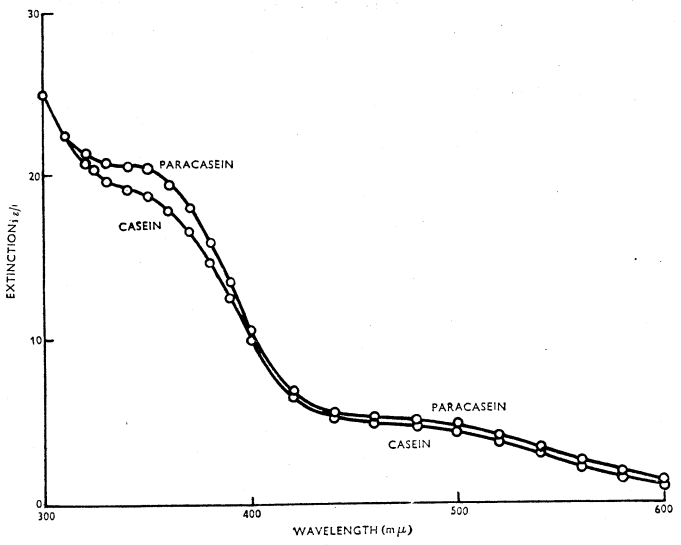

Fig. 4.—Absorption spectra of azo-derivatives of casein and paracasein (0.01 g/25 ml).

hydrolysates were first neutralized with sodium hydroxide, and ethanol was omitted from the coupling media because it increases the instability of azo-amino derivatives. The spectra obtained are shown in Figure 6 for a concentration at which all the potentially reactive groups in the hydrolysates can react with the diazonium salt. The data of Figures 4, 5, and 6 have been converted to a protein concentration of 1 g/l, but no correction has been made for other absorbing components, such as excess reactants. The only appreciable error thus introduced will result from the presence of unreacted *p*-diazobenzenesulphonic acid.

As the interpretation of these data involves the amino content of the proteins and their hydrolysates, the amino groups were determined, with the following results (expressed as equivalents $NH_2/10^5$ g air-dry protein): intact casein 43.7; intact paracasein 47.0; casein hydrolysate 665; paracasein hydrolysate 690.

(*e*) *Clotting of α-Casein*

The following experiment was carried out to determine the relative responses of whole casein and α-casein towards rennet treatment. To 5-ml aliquots of 1 per cent. solutions of each (on an air-dry basis), adjusted to pH 6.64, were added 0.05 ml rennet solution and 0.2 ml 1M calcium chloride solution. Clots formed after *c.* 10 min at 25°C. The supernatants were filtered off through

sintered glass and their extinctions measured against water at 277 mμ. They were:

Whole casein 1.99;
α-Casein 1.52.

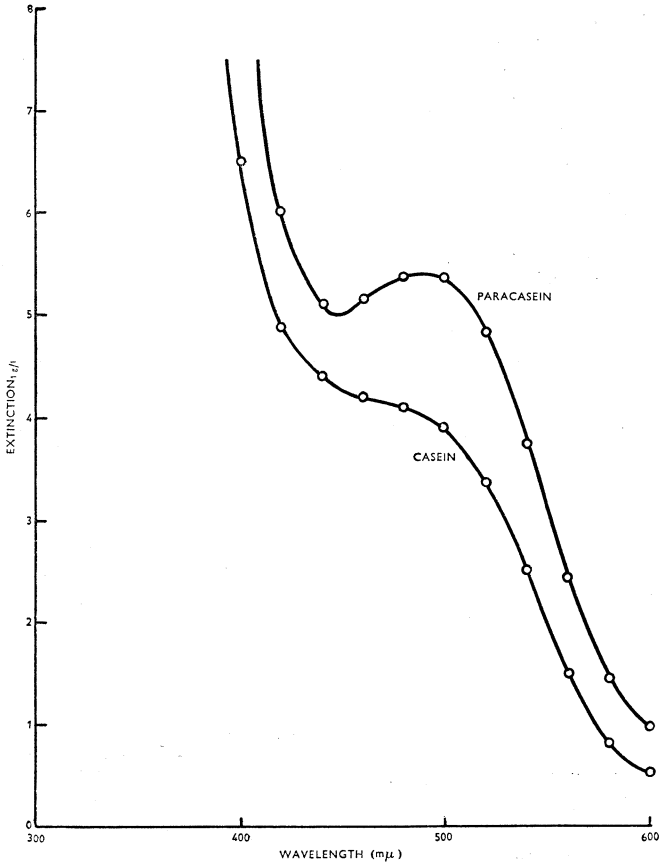

Fig. 5.—Absorption spectra of azo-derivatives of casein and paracasein (0.01 g/25 ml).

(*f*) *Effect of Ionic Strength and Anion Valency on Rate of Clotting*

Experiments based on the investigations of Lovelock and Porterfield (1952) on the clotting of blood plasma were carried out with paracasein. The solutions, of final concentration 3 g/100 ml adjusted to pH 6.4 with sodium hydroxide, were maintained at 25°C under the various conditions shown in Table 3, which indicates whether or not a clot formed 30 min after the addition of the calcium chloride. It was observed that, where clots formed in the presence of potassium ferricyanide, the process was almost instantaneous and they were more rigid and coherent than those formed in the presence of electrolytes of lower anion valency.

(g) *Stability of the Clots*

In order to obtain further information on the nature of the intermolecular bonds within the rennet-sponsored clots, the stability of the clots was examined under various conditions. To three 10-ml aliquots of a 3 per cent. solution of paracasein of pH 6.7, 1 ml 1M calcium chloride was added, the clots were allowed to form, and the supernatants were removed. Five ml of the reagents shown in Table 4 were added, one to each clot, with the results indicated.

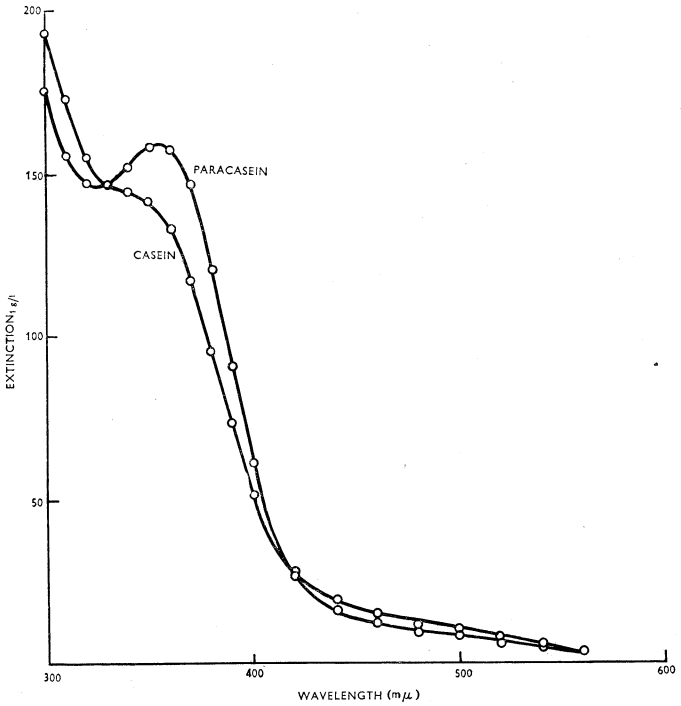

Fig. 6.—Absorption spectra of acid hydrolysates of casein and paracasein, after reaction with diazotized sulphanilic acid (0.01 g intact protein/25 ml).

IV. Discussion

The interpretation of the titration data is by no means unequivocal, but they assist in evaluating the change brought about in the casein upon rennet treatment. The electrometric titration curve shown in Figure 1 shows the greater binding capacity of the paracasein above pH 6.8 (also shown by its lower equilibrium pH value in the presence of a given quantity of sodium hydroxide) and between pH 5.2 and pH 3.2.

If the ethanol and acetone titrations are interpreted according to Lea and Hannan (1950), who were working with casein, it can be concluded from Table 2 that the increase in basic groups upon rennet treatment is greater by six than that in the acid groups. This cannot be ascribed to α-amino (since

the appearance of each group of this type would be accompanied by a corresponding carboxyl), to ε-amino (from the formol titration), or to guanidino (since arginine is not determined) and so attention can be directed to the

TABLE 3

EFFECT OF IONIC STRENGTH AND ANION VALENCY ON CALCIUM CHLORIDE CONCENTRATION REQUIRED FOR CLOTTING

Crosses indicate clotting; dashes no clotting

Electrolyte		Sodium Chloride				Potassium Ferricyanide			
Molarity		0·1	0·2	0·3	0·4	0·1	0·2	0·3	0·4
Ionic Strength*		0·1	0·2	0·3	0·4	0·6	1·2	1·8	2·4
Final molarity of calcium chloride	0·018	+	−	−	−				
	0·045	+	−	−	−				
	0·072	+	+	+	−				
	0·090	+	+	+	−	−	−		
	0·18					−	−	−	−
	0·27					−	−	−	−
	0·38					−			+
	0·45	+	+	+	+	+	+	+	+
	0·91					+	+	+	+

* Taking molarity as molality and assuming complete ionization.

imidazole group of histidine. Richardson (1934) maintained that the scission of ester bonds will be estimated by the alkaline titration but not by the acid, and this could also account for the observed differences if his interpretation is correct.

TABLE 4

STABILITY OF CLOTS

Reagent	Result
1N NaOH	Clot dissolved rapidly
1N HCl	Clot did not dissolve
Saturated NH_4Cl	Clot dissolved slowly

The observation that paracasein, after removal of the proteose-containing supernatant and re-solution, could still be coagulated suggests that the liberated proteose is not essential for clotting, a view also favoured by Cherbuliez and Baudet (1950), although the possibility remains that a substance in the proteose is strongly adsorbed on the paracasein. The possibility that liberation of the

proteose exposes functional groups which can then react with calcium ions is also not precluded.

The relatively low solubility of paracasein at high protein concentration may be due to salting out by a small component or to complex formation between components of the protein mixture.

The differences in the ultraviolet absorption of casein and paracasein suggest that rennet may affect the absorbing capacity of the tyrosine groups, perhaps by rupturing intermolecular hydrogen bond structures involving the phenolic hydroxyl, and a detailed spectrophotometric study of this question might well be warranted. Paracasein exhibits greater coupling capacity with diazonium salts than casein, as shown by the higher extinctions of the azo-derivatives (Figs. 4 and 5). The absorption in the region 480-500 mμ can be related to the imidazole or phenolic hydroxyl groups (Higgins and Fraser 1952) and that at *c.* 360 mμ to amino groups (from unpublished data of the authors). The spectra of the azo-derivatives of the hydrolysates (Fig. 6) do not reveal a higher histidine or tyrosine content in the paracasein, as shown by extinctions at 480-500 mμ and at 325 mμ, and differences in the intact protein thus appear to be related to the accessibility of the groups concerned.

The suggestion that rennet may release some of the imidazole rings from intramolecular linkages is in accordance with the indirect indications of the ethanol and acetone titrations. D'yachenko (1950) also refers to the release of the imidazole ring during rennet fermentation, on the basis of potentiometric titration data, although he does not assign to this reaction a subsequent role in the clotting process.

According to the definition of denaturation given by Neurath *et al.* (1944), paracasein may perhaps be considered as a denatured protein in that the reactivity of certain side chains is apparently increased by intramolecular modification. On the other hand, the striking increase in solubility (in the absence of calcium ions) and the maintenance of molecular symmetry, as determined by viscosity measurements (D'yachenko 1950; Higgins and Hayes 1951), upon rennet treatment of casein are changes in the opposite sense to those usually encountered in denaturation processes. Like casein, paracasein also yields negative sulphydryl tests.

The data on the clotting of α-casein and whole casein suggest that the α-casein enters more fully into the structure of the clot, which supports the idea that the α-casein is more affected by the rennet than the other components. This result is in accordance with those of Pyne (1951), who found that, after rennet treatment, whole casein required a higher calcium ion concentration than α-casein to effect the same rate of coagulation.

The variation with ionic strength of the amount of calcium required for coagulation in the presence of sodium chloride shows that a certain electrolyte balance is necessary for clotting, as for blood plasma (Lovelock and Porterfield 1952). Whether, as these authors consider is the case for plasma, the function of the calcium is to maintain the surface charge of one or more of the colloidal components at a value suitable for interaction with the others, or whether D'yachenko and Nitschmann and Lehmann are correct in regarding the calcium ions as acting as bridges between paracaseinate molecules, is still open to doubt.

Although potassium ferricyanide requires a higher calcium ion concentration for clotting than sodium chloride, on a molar basis, the clots form almost instantaneously when the electrolyte balance is suitable and are more rigid and coherent. A possible explanation of this behaviour is that the trivalent anion facilitates three-dimensional cross-linking through calcium bridges attached to acid groups in different protein molecules.

The observations on the stability of the clots, considered in conjunction with the data discussed above, seem to be compatible with the formation of intermolecular bonds of a predominantly ionic character.

V. Acknowledgments

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