XII. ON THE ACTION OF COAGULATING ENZYMES ON CASEINOGEN.

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Hammarsten [1872, 1874, 1877] first demonstrated that the rennin action on caseinogen was specific and independent of the action of the calcium salts. His explanation was that the caseinogen molecule was split up into a large molecule (Käse) and a smaller one (Molkeneiweiss). The "Käse" was rendered insoluble by the presence of soluble calcium salts and formed the clot. Since then little has been done to determine the chemistry of the clotting process, and our knowledge of this branch of the subject has up till recently been untouched by investigators.

The recent literature contains views which contradict the theory advanced by Hammarsten. Schryver [1913, 1 and 2] and Mellanby [1913] both consider that the rennin clot is probably a combination of enzyme and protein, and Schryver states definitely that rennin alone causes no proteoclastic change. Bosworth [1913] has found that the rennin does not split off any nitrogen from the caseinogen which remains in solution when the casein is precipitated by dilute acetic acid. The protein molecule has therefore undergone no cleavage into its components.

This, considered in connection with the results of his earlier work with van Slyke [1913], leads him to believe that the ferment breaks up the caseinogen molecule into two molecules of casein each half the size of the original molecule. In the case of basic calcium caseinogenate (containing 4 equivalents of calcium) the casein produced is soluble in water but is rendered insoluble by the presence of small quantities of calcium chloride. The caseinogenate containing two equivalents of calcium gives a casein insoluble in water¹.

¹ The English nomenclature—caseinogen for the unfermented protein and casein for the product of fermentation—is used throughout.

ENZYMES EMPLOYED.

The enzymes used were Witte's rennin powder 1:300,000, Grüber's trypsin, and a preparation made from the seeds of the *Withania coagulans* [Lea, 1884].

The rennin preparation was used in neutral aqueous suspension, was exceedingly active and fermented in a short time when the concentration was as low as 1:500,000. In the experimental work the concentration employed was 1 of rennin to 300,000 of caseinogen solution. The trypsin was used in aqueous suspension, in the proportion of 1 part of the solid preparation to 12,000 of caseinogen solution. The *Withania coagulans* enzyme was prepared by grinding up the seeds to a fine powder and extracting with 5 per cent. sodium chloride solution. Three volumes of absolute alcohol were added and the ferment precipitated. It was filtered, washed with absolute alcohol and dried at 37°. One gram of the powder thus obtained was made up to 100 cc. with 5 per cent. sodium chloride solution; 5 cc. of this solution were used to ferment 100 cc. of caseinogen solution. The trypsin and rennin contained no calcium or phosphorus estimable in three grams of the dried preparation, while the vegetable rennin contained a trace of phosphorus and no calcium.

PREPARATION AND PROPERTIES OF THE CASEINOGEN.

At the commencement of the investigation it was found that the commercial preparations of caseinogen, even when obtained from reliable sources, did not give satisfactory results. In fact the rennin had scarcely any appreciable effect on them. A modification of Hammarsten's method was finally adopted which gave satisfactory caseinogen preparations, Schryver's plan of drying in different grades of alcohol being employed.

The method was as follows:—skimmed milk containing 0·1 per cent. of fat was taken, diluted with five times its volume of distilled water and 0·1 per cent. of acetic acid added slowly, the whole being constantly agitated until the protein separated out. The caseinogen was then allowed to settle and the supernatant fluid syphoned off. The mixture of caseinogen and diluted milk serum was poured on a cheese cloth filter and the remaining fluid drained off. The caseinogen was then washed by mixing it with a volume of distilled water equal to the quantity of milk from which it was obtained and shaking thoroughly. When settled the water was again syphoned off and the caseinogen washed a second and third time with the same volume of distilled water. It was next redissolved by shaking with 0·2 per cent. sodium carbonate

solution and the precipitation and washing carried out as before, the solution, precipitation and washing being repeated at least twice after the original precipitation from milk. Finally it was ground up successively with 33 per cent., 66 per cent. and absolute alcohol and given two half-hour extractions in an agitator with ether, filtered and dried for two hours at 37°.

These preparations upon analysis were found to be completely free from fat and had a phosphorus content of 0·87–0·90 per cent. The ash from 10 g. contained no estimable calcium. Solutions were prepared by grinding the dried powder with moist calcium carbonate in a mortar, centrifuging and filtering to remove the excess of calcium carbonate, the caseinogen when treated in this way having a solubility of 19–20 cc. (5 cc. gave by Kjeldahl's method enough ammonia to neutralise this volume of N/10 acid). The solutions were opaque and milky in appearance, neutral to litmus and contained the amount of calcium necessary to form the basic caseinogenate. After treatment by rennin these solutions readily gave precipitates when about 5 per cent. by volume of N calcium chloride was added. This amount of calcium chloride alone with the original caseinogen solutions produced no precipitate from 0°–40°, but when the temperature was raised to 50°–60° a precipitate was formed. The precipitates produced by the action of enzyme and calcium chloride rapidly contracted to a small fraction of the volume of the solution, while those produced by calcium chloride and heat settled very slowly and did not shrink in volume.

COMPOSITION OF CASEINOGEN AND CASEIN.

The experiments were carried out as follows: 200 cc. portions of caseinogen solution were put into beakers and the enzymes added in the proportions already mentioned. Control experiments were made, using equal amounts of caseinogen solution and enzyme solution inactivated by boiling, and submitting these to the same conditions as those containing the active enzyme. The mixtures were placed in an incubator at 37° for one hour, after which 10 cc. N calcium chloride (5·5 g. per 100 cc.) were added both to controls and fermented preparations. In the case of the fermented solutions the precipitate at once came down. Precipitates from the controls were obtained by heating them to 55°. When the precipitates had settled the supernatant liquid was decanted and the precipitates thoroughly broken up and shaken vigorously with 500 cc. of 10 per cent. alcohol in a tall cylinder. When they had again settled the clear fluid was syphoned off and this process repeated three times.

The last washing was found to be free from chlorides. To inactivate the enzyme the precipitates were mixed with water and boiled for five minutes, the controls being similarly treated. All the precipitates were finally ground up with absolute alcohol and ether and dried at 37°.

The nitrogen, calcium, phosphorus ratios were determined as an index of composition, nitrogen being taken as the fixed unit of comparison. Phosphorus was estimated by Neumann's method and nitrogen by Kjeldahl's. Calcium was estimated by ashing the dried caseinogen or casein, dissolving the ash in hydrochloric acid and neutralising with ammonia until the phosphates were precipitated. These were redissolved by adding strong acetic acid, and the calcium precipitated as the oxalate and weighed as the sulphate. No phosphorus was precipitated by this method.

The results are given in the following tables.

I. *Rennin action.*

Caseinogen (controls)				Casein			
	N	P	Ca		N	P	Ca
1.	100	5.8	10.4	3.	100	5.65	9.60
2.	100	5.8	10.5	4.	100	5.60	9.53
				5.	100	5.45	9.64
				6.	100	5.65	9.66

II. *Trypsin action.*

Caseinogen (controls)				Casein			
	N	P	Ca		N	P	Ca
7.	100	5.54	12.41	9.	100	4.85	10.42
8.	100	5.50	12.15	10.	100	4.32	10.17
				11.	100	4.71	10.47

III. *Withania coagulans.*

Caseinogen (controls)				Casein			
	N	P	Ca		N	P	Ca
12.	100	5.58	12.58	14.	100	5.48	12.40
13.	100	5.61	12.62	15.	100	5.45	12.35

These series of analytical results demonstrate clearly that the enzyme action results in some proteoclastic change in the caseinogen molecule. The first series on rennin action was carried out with a preparation of caseinogen which was different from that used in the other two series and had a somewhat higher phosphorus content. Two criticisms of these results may be advanced—that it may not be possible to remove all the absorbed calcium from the casein and that boiling the precipitates to inactivate the enzyme may split off phosphorus, a possibility which Schryver points out. With regard to the first point it has been found that the final washing is free from chlorides and that a uniformly lower calcium content appears in casein throughout the series. To settle the second point caseinogen was taken,

fermented by rennin and calcium chloride added. The precipitate was filtered off and the N:P ratio was determined in the filtrate and compared with that given by a caseinogen solution to which equal amounts of calcium chloride and inactive enzyme had been added under the same conditions. In no case was the temperature higher than 37°, so that any difference observed must be due to enzyme action.

Caseinogen (controls)			Filtrate from casein	
	N	P	N	P
1.	100	5.76	3.	100
2.	100	5.80	4.	100
			5.	100
			6.	100
				12.2
				12.0
				10.7
				13.1

This clearly demonstrates that the phosphorus cleavage was due to the rennin action alone and not to the boiling with water.

These figures for caseinogen are in agreement both in phosphorus and calcium content with most of the preparations of the basic salt quoted in the literature published on this subject.

On the other hand the casein shows a slightly lower phosphorus or calcium content relative to the nitrogen. These figures are not comparable with the phosphorus or calcium content of the acid salts of casein described by other investigators. Schryver finds that no change in the N:P ratio takes place when caseinogen is converted into casein, and van Slyke and Bosworth [1913] have prepared a basic caseinogenate and a caseinate from milk of the same phosphorus and calcium content.

The cleavage is most marked in the case of trypsin where from 12-20% of the phosphorus is split off, the action thus bearing a close resemblance to that exerted by this enzyme in alkaline solution [Plimmer and Bayliss, 1906]. The action is less marked in the case of the Withania enzyme.

ENZYMIC PRECIPITATION OF CASEINOGEN SOLUTIONS WITHOUT THE ADDITION OF CALCIUM SALTS.

Furthermore, caseinogen solutions after being subjected to ferment action give precipitates without addition of calcium chloride if the ferment be present in sufficient concentration. Caseinogen can thus be precipitated without the addition of calcium chloride in twenty-four hours by rennin when the latter is present in the proportion of 1 part of dried ferment to 1000 of solution. Trypsin in three to four hours also forms a precipitate in 1:5000 solution without the addition of calcium chloride. Withania coagulans also produced a small precipitate without addition of a soluble calcium salt in four

hours when one volume of ferment solution was added to five of caseinogen solution. Again, when the ferment was more dilute (rennin 1:100,000, trypsin 1:15,000, Withania 1:20) precipitates appeared when the temperature was raised from 45°–50° even when the ferments had acted only for twenty minutes and in the absence of added calcium salts. Controls containing the same concentrations of inactivated enzyme did not give these reactions.

NATURE OF THE CLEAVAGE PRODUCTS.

The filtrates from casein preparations were taken and examined to ascertain the nature of the nitrogen and phosphorus split off. Cathcart's precipitating mixture as used by Plimmer and Bayliss [1906] was added to casein solutions to precipitate the protein and its higher cleavage products, and analyses were made on aliquot parts of the filtrate. In the case of the casein produced by rennin action no soluble phosphorus or nitrogen was found. No inorganic phosphorus was produced by any of the three ferments.

100 cc. of caseinogen solution were taken containing 0.521 g. N and 0.0295 g. P. N:P = 100:5.6.

Produced from 100 cc. caseinogen solution and not precipitated by tannic acid			
	Soluble N	Soluble P	Ratio N:P
Trypsin action	1. 0.0203 g.	0.0033 g.	100:16.2
	2. 0.0084 g.	0.0015 g.	100:17.8
Withania action	3. 0.0028 g.	0.0006 g.	100:21.4
	4. 0.0022 g.	0.0009 g.	100:40.9

Trypsin as shown by these analyses gives the largest quantities of soluble nitrogen and phosphorus, and splits off some phosphorus group relatively rich in phosphorus, a N:P ratio considerably higher than the normal being obtained. In the case of the Withania enzyme the phosphorus is still higher in relation to the nitrogen but the total quantities of soluble nitrogen and phosphorus are very small. The amounts given above probably do not represent the total cleavage, some of the products in each case being presumably in a form precipitable by tannic acid.

As already mentioned the behaviour of trypsin in converting caseinogen into casein in neutral solution is somewhat like its proteoclastic action in alkaline solution, except that no inorganic phosphorus is produced as in alkaline solutions [Plimmer and Bayliss, 1906]. The effect of the trypsin used when allowed to act in alkaline solutions is shown by the following experiment.

Caseinogen solution and trypsin solution were taken in the same quantities as for the precipitation experiments, and enough alkali to make the concentration 0.2 per cent. NaOH added. This was incubated at 37° for three hours. Part was taken, the protein precipitated with dilute acetic acid and the inorganic phosphorus precipitated from the filtrate by magnesium citrate mixture, the phosphorus being estimated by Neumann's method in the phosphate precipitate. In another portion the soluble phosphorus was estimated as previously described.

100 cc. original solution.	Total P = 0.0268 g.
100 cc. solution after trypsin action.	Soluble P = 0.0150 g.
100 cc. solution after trypsin action.	Inorganic P = 0.0043 g.

THE IRREVERSIBILITY OF THE CHANGE.

Caseinogen when precipitated from solution in the presence of calcium chloride at 55°, washed with 10 per cent. alcohol, redispersed in caustic soda, again precipitated with acetic acid, washed and dissolved by calcium carbonate gave solutions not nearly as concentrated as those obtainable from the original material. These latter had concentrations of 17–19.5 cc., but after the above process products were obtained, the solubilities of which were only from 7–13 cc. These could readily be acted on by any of the enzymes and a characteristic casein precipitate obtained.

Casein after the above treatment had very low solubilities, ranging from 0.5–4 cc. and the solutions were transparent, and when submitted to further enzyme action did not yield products more readily precipitated by calcium chloride than themselves.

This confirms the observations of Hammarsten and Schryver on this point.

PRECIPITATION OF CASEINOGEN AND CASEIN BY ELECTROLYTES.

The precipitating action of a number of salts was tested on caseinogen and casein solutions prepared by means of calcium carbonate as described. 5 cc. of solutions exposed to ferment action for one hour and the same amount from controls containing inactivated ferment were diluted to 100 cc., boiled, cooled and 1 cc. quantities put in test tubes in which 1 cc. of the various salt solutions had been previously placed. The concentrations indicated in the tables are those of the salts in the final mixture. The mixtures were allowed to stand at room temperature for one hour; + indicates a precipitate.

TABLE I.

1. *Rennin action.*

Concentration of salt in final mixture	CaCl ₂		BaCl ₂		MgCl ₂		SrCl ₂	
	Casein- ogen	Casein	Casein- ogen	Casein	Casein- ogen	Casein	Casein- ogen	Casein
2N	-	-	-	-	-	-	-	-
N	-	+	-	+	-	-	-	-
N/2	-	+	-	+	-	-	-	+
N/4	-	+	-	+	-	+	-	+
N/10	-	+	-	+	-	+	-	+
N/20	-	+	-	+	-	+	-	+
N/40	-	+	-	+	-	+	-	-
N/80	-	-	-	-	-	-	-	-

2. *Trypsin action.*

2N	-	-	-	-	-	-	-	-
N	-	+	-	+	-	-	-	-
N/2	-	+	-	+	-	+	-	-
N/4	-	+	-	+	-	+	-	+
N/10	-	+	-	+	-	-	-	+
N/20	-	+	-	+	-	-	-	-
N/40	-	-	-	-	-	-	-	-
N/80	-	-	-	-	-	-	-	-

3. *Withania coagulans action.*

2N	-	-	-	-	-	-	-	-
N	-	-	-	-	-	-	-	-
N/2	-	+	-	+	-	-	-	-
N/4	-	+	-	+	-	-	-	+
N/10	-	+	-	+	-	+	-	+
N/20	-	+	-	+	-	+	-	-
N/40	-	-	-	-	-	-	-	-
N/80	-	-	-	-	-	-	-	-

Calcium and barium salts in the case of all the enzymes are most effective as precipitating agents. The ranges of the action of strontium and magnesium salts are similar, but are narrower than in the case of calcium and barium salts. This holds for all the enzymes with slight individual variations. In no case was caseinogen precipitated within the range employed.

In the case of the lanthanum salt (Table II, p. 98) the requisite concentration is very low, being N/800 for caseinogen. This is in accord with the findings of other workers, the trivalent ion being an exceedingly powerful one as a precipitating agent. Again the variation for the different enzymes is seen, but to a greater extent, especially for casein obtained by trypsin action.

The variation in the different caseins is probably due to the influence of the cleavage products present in the mixture which, as shown earlier, are different for each ferment.

TABLE II.

Precipitating action of lanthanum nitrate.

Concentration of salt in final mixture	Rennin action		Trypsin action		Withania action	
	Caseinogen	Casein	Caseinogen	Casein	Caseinogen	Casein
N/50	-	-	-	+	-	-
N/100	-	-	-	+	-	-
N/200	-	-	-	+	-	-
N/400	-	+	-	+	-	-
N/800	+	+	+	++	+	+
N/1600	-	++	-	++	-	++
N/3200	-	-	-	-	-	-

It is remarkable that both caseinogen and casein are precipitated by the same concentration of lanthanum nitrate, whereas they require very different concentrations of calcium, barium, strontium and magnesium salts.

The investigation of the precipitating action of rennin on caseinogen in milk is an extremely difficult problem, since here the reaction of the liquid and the presence of salts, carbohydrates and fats all play a part which may obscure the essential factors of the process. For this reason solutions of pure caseinogen have been used throughout the foregoing experiments. Another point which is of the greatest importance is the method of preparation of the caseinogen. The different methods used for this purpose probably account for much of the diversity of opinion which exists at present on the nature of the enzyme action. In our preparations from milk the use of sodium hydrate was avoided so as to escape the possibility of hydrolysing the protein. Van Slyke and Bosworth have used ammonia in the final stages of their method, allowing it to remain in contact with the caseinogen overnight. Their preparations have a very low ash content and the phosphorus content is the lowest yet recorded. This is due possibly to removal of phosphorus by the action of the alkali.

The foregoing results indicate that the substance precipitated by calcium chloride from solutions of caseinogen, which have been submitted to the action of rennin, trypsin or Withania enzyme, differ not only in precipitability by salts and solubility but also in chemical composition.

CONCLUSIONS.

- (1) The conversion of caseinogen into casein by enzyme action is accompanied by the cleavage of nitrogen, phosphorus and calcium.
- (2) Rennin action produces no soluble nitrogen or phosphorus. Trypsin splits off both soluble nitrogen and phosphorus, while the Withania enzyme

also produces soluble nitrogen and phosphorus but in smaller absolute quantities.

(3) The cleavage products are specific for each enzyme and it is to this difference of enzyme action that the variation in behaviour of the resulting casein is to be ascribed.

(4) The precipitation of calcium caseinate by soluble calcium salts is not due to any chemical combination with these.

(5) The caseinogen once exposed to enzyme action and redispersed cannot be rendered more precipitable by renewed enzyme action.

(6) If the enzyme be sufficiently concentrated, precipitates are obtained without the addition of calcium salts and the same thing occurs with more dilute enzyme solutions when the temperature is raised above 45°.

In conclusion we acknowledge our indebtedness to the Director of the Imperial Institute for the supply of seeds of *Withania coagulans* from which the enzyme was prepared.

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