XXXV. THE PREPARATION AND COMPOSITION OF CASEINOGEN.

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Definition. In this paper the word *caseinogen* denotes the main protein present in fresh milk; *acidic caseinogen* the protein precipitated from milk by acid; and *casein* the protein precipitated from milk by the action of proteolytic ferments and calcium salts. The experimental facts upon which this terminology is based are set forward in the succeeding pages.

Introduction. The experiments described in this paper were made on caseinogen prepared directly from fresh milk by alcohol precipitation. A considerable quantity of work has been done on the protein precipitated from milk by the addition of acid to it (acidic caseinogen) on the assumption that this substance is the basic protein of milk. The prevalent hypothesis as to the relation of caseinogen to acidic caseinogen is founded upon an experiment of Ringer [1890] in which he showed that when a suspension of acidic caseinogen in water is triturated with calcium carbonate, the protein is dissolved forming a milk white solution. This experimental result is explained on the assumption that acidic caseinogen is an acid which reacts with calcium carbonate forming calcium caseinogenate (assumed to be identical with the caseinogen of milk) and liberating carbon dioxide thus:

The precipitation of acidic caseinogen from milk by the addition of small quantities of acid is expressed by the equation

In the following pages evidence is brought forward in favour of the view that the main protein of milk consists of a complex of protein and calcium phosphate in definite proportions, and that the protein precipitated from milk by the addition of acid (acidic caseinogen) is different from that contained in the caseinogen complex. My conclusions are at variance with those

of other workers in so far as they have assumed that acidic caseinogen is essentially the same as caseinogen and have used the former protein as the basis of their experimental work. I propose to defer the consideration of the properties of caseinogen and the relation of acidic caseinogen to it.

The preparation of caseinogen from milk. In a previous paper [1907] I have shown that the precipitation of a protein from solution by alcohol usually involves two distinct processes; (i) precipitation of the protein; (ii) coagulation of the protein, this latter process rendering the precipitate insoluble in the original solvent. In the case of the protein of horse serum these two processes can be readily dissociated by precipitation above or below 14° C. Below this temperature, alcohol, added to the extent of 60 %, precipitates the protein in an unchanged condition; above this temperature the protein is not only precipitated but also rendered insoluble, the degree of insolubility of the precipitated protein being a function of (a) the temperature of precipitation, (b) the amount of alcohol used and (c) the length of time during which the precipitated protein is left in contact with the alcohol. These actions of alcohol are dependent upon the concentration of electrolytes contained in the protein solution, the less the ionic energy per unit volume the more effective the precipitating action of a definite quantity of alcohol, and the less insoluble the resulting precipitated protein. Similar facts hold good for the protein of milk. If alcohol, to the extent of 50 %, be added to fresh milk, caseinogen is not only precipitated but also coagulated. If, however, alcohol be added to the extent of 75 % to milk cooled below 15° C., the resultant protein precipitate, when freed from alcohol, is readily soluble in water.

The following method was used for the precipitation of caseinogen from milk by alcohol.

To one volume of separated milk three volumes of alcohol were added, both the milk and the alcohol being cooled below 15° C. The precipitate, after being strained through muslin and so freed from excess of alcohol, was ground up in a mortar with ether, filtered, and again extracted with ether until freed from fat and pigment. The final product was pressed between folds of filter paper and allowed to dry in the air. It is convenient to use fresh separated milk since milk fat accompanies caseinogen in the alcohol precipitation, and a large quantity of fat requires a large quantity of ether for its extraction. Also it is not advisable to cool the alcohol to 0° C. since at this temperature a large quantity of lactose is precipitated with the caseinogen.

The precipitate obtained from milk by alcohol consists of caseinogen admixed with small quantities of lactose, lactalbumin, and inorganic salts. The solid is not dry in the sense that it does not lose weight when heated to 100° C. It is advisable to keep it in an impure condition in a stoppered bottle since if heated to 100° C. in an air bath it passes into a modification insoluble in water.

The composition of caseinogen. The composition of caseinogen was determined by making a series of experiments on the impure substance. The caseinogen was not separated from all impurities before determining its properties since such a procedure involves prolonged manipulation, and caseinogen being a labile protein readily passes into modifications which differ from the original substance. The crude substance when suspended in water at 37° C. forms a milk white solution. This solution takes place slowly (about thirty minutes) probably owing to the difficulty of the water getting into intimate contact with the large protein aggregates. An important fact to notice, however, is that the crude substance contains no substances insoluble in water such as calcium phosphate, or a protein comparable in solubility to acidic caseinogen.

The impurities associated with caseinogen in the crude alcoholic precipitate can be estimated by taking advantage of their solubilities compared with that of caseinogen. Caseinogen is practically insoluble in water at 15° C., whilst lactose and lactalbumin are freely soluble at this temperature. Therefore in order to determine the amount of caseinogen contained in five grams of the solid, this quantity of substance was suspended in water, made up to 100 cc., and allowed to extract at room temperature (15° C.) for twenty hours. At the end of this time the suspension was filtered, and the weight of solid in a measured quantity of the filtrate determined by drying over a water bath and completing the drying in an air bath at 120° C. for four hours.

The following figures give the results of such an analysis for one gram of the impure solid:

Weight of substance soluble in water at 15° C.			
contained in 20 cc. of filtrate	...	...	0.19 g.
Loss in weight of 1 g. of solid after drying at			
120° C. for four hours (H ₂ O, etc.)	...		0.202 g.
			<u>0.392 g.</u>

Therefore one gram of the solid contained 0.608 g. of caseinogen.

(a) *The calcium and phosphorus content of caseinogen.* The amount of calcium contained in caseinogen was determined by the method of Hurtle and Kahn [1915]. The method consists of ashing a known weight of caseinogen, dissolving the ash in a small quantity (5 cc.) of 25 % phosphoric acid, diluting to 100 cc. with water, precipitating the calcium by slowly adding from a burette 100 cc. of 5.524 % potassium oxalate, washing the precipitate with water till free from soluble oxalate, dissolving it in dilute sulphuric acid, and determining the quantity of freed oxalic acid (and consequently calcium) by titration with tenth normal permanganate. The following figures give the results of a typical experiment made in this way.

Calcium contained in one gram impure, undried caseinogen

$$= 12.8 \text{ cc. KMnO}_4 (0.1 \text{ N}).$$

The calcium contained in the substances soluble in water at 15°C. = 3.08 cc. KMnO_4 (0.1 N).

From the figures previously given the weight of caseinogen contained in one gram of impure substance = 0.608 g.

Therefore the amount of calcium contained in one gram of pure caseinogen = 15.84 cc. KMnO_4 (0.1 N) = 0.0317 g. calcium.

The amount of phosphorus contained in the caseinogen was determined by a modified Neumann's [1902] method. Amounts of impure caseinogen varying in weight from 0.2 g. to 0.5 g. were oxidised by the method described by Plimmer and Bayliss [1906]. In this oxidation it was necessary to use small quantities of the protein and prolonged heating with nitric acid to ensure complete destruction of the protein. The resultant fluid was diluted to 100 cc. and after heating to 80°C., 100 cc. of a phosphate precipitating mixture was added, and the heating continued on a water bath for 15 minutes. For precipitation of the phosphate a filtered mixture of ammonium nitrate and ammonium molybdate in nitric acid made up in the proportions recommended by Halliburton [1914] was used.

This solution does not deposit molybdic acid in the cold but when heated to 100°C. the white modification of molybdic acid separates out. The precipitation of molybdic acid is prevented by sulphuric acid, and it was essential therefore so to conduct the preliminary hydrolysis of the protein that sufficient sulphuric acid remained in the hydrolysed fluid to prevent the precipitation of molybdic acid, but not that of the ammonium phosphomolybdate, after the addition of the precipitating mixture, and subsequent heating on a water bath. The following gives the experimental

results obtained from the same sample of crude caseinogen as was used in the experiments above.

P_2O_5 in one gram impure caseinogen = 38.5 cc. NaOH (0.5 N).

P_2O_5 contained in the substances soluble in H_2O at $15^\circ C.$ = 9 cc. NaOH (0.5 N).

Therefore the P_2O_5 contained in the caseinogen was equivalent to 29.5 cc. NaOH (0.5 N).

The weight of caseinogen contained in one gram of impure substance = 0.608 g.

Therefore the amount of P_2O_5 contained in one gram of pure caseinogen = 48.6 cc. NaOH (0.5 N) = 0.062 g. P_2O_5 .

(b) *The calcium and phosphorus contents of acidic caseinogen.* On the addition of acid to a solution of caseinogen a protein (acidic caseinogen) is precipitated. A comparison of the calcium and phosphorus contents of caseinogen and acidic caseinogen yields interesting results. The acidic caseinogen used in these experiments was prepared by dissolving the impure caseinogen in water, precipitating the acidic caseinogen by the addition of an optimal quantity of acetic acid and washing the precipitate with water, alcohol and ether. It may be remarked that acidic caseinogen prepared in this way is identical in phosphorus content with that prepared by the acidic precipitation of fresh milk, and with that of Merck's "pure casein" (acidic caseinogen) prepared by Hammarsten's method. The identity of the results obtained with these specimens of acidic caseinogen affords presumptive evidence in favour of the view that caseinogen precipitated from milk by alcohol is the protein of milk in an unaltered condition.

Acidic caseinogen prepared from caseinogen contains no calcium and in this respect also agrees with the analysis of acidic caseinogen obtained from milk by Hammarsten's method. The absence of calcium is in marked contrast to the large quantities of this element contained in caseinogen.

The phosphorus content, however, although less than that of caseinogen, is large. The following figures give the average of a series of experiments.

P_2O_5 in one gram acidic caseinogen (pure) = 16.5 cc. NaOH (0.5 N) = 0.021 g. P_2O_5 .

(c) *A comparison of the calcium and phosphorus contents of caseinogen and acidic caseinogen.* From the figures given above it may be seen that one gram of caseinogen contains 0.0317 g. calcium and 0.062 g. P_2O_5 , and one gram of acidic caseinogen contains no calcium and 0.021 g. of P_2O_5 .

The question arises as to whether these figures give any indication of

the relation of caseinogen to acidic caseinogen, and the manner in which calcium is contained in the caseinogen complex. We may legitimately assume that the phosphorus contained in acidic caseinogen is an organic combination since there are no inorganic bases to combine with it. If this amount of phosphorus be organically contained in caseinogen also, there is now 0.0406 g. P_2O_5 contained in one gram of caseinogen not accounted for. On the assumption that the calcium is associated with the residual P_2O_5 (0.0406 g.) in the caseinogen complex as $Ca_3(PO_4)_2$, 0.034 g. of calcium would be required. The approximation of the theoretical quantity of calcium required (0.034 g.) with that actually determined (0.0317 g.) offers strong evidence in support of the hypothesis that in caseinogen the phosphorus is present in two forms (a) in organic combination, (b) associated with calcium as calcium phosphate.

(d) *A comparison of the quantity of phosphorus contained in acidic caseinogen with that of inorganic phosphorus contained in caseinogen.* The amount of phosphorus contained in one gram of acidic caseinogen is 0.021 g. P_2O_5 . On the assumption that a molecule of acidic caseinogen contains at least one atom of phosphorus, the least molecular weight for caseinogen is 3375, i.e. 3375 g. of acidic caseinogen contain one gram atom of phosphorus.

The amount of inorganic phosphorus assumed to be present in one gram of caseinogen is 0.0406 g. P_2O_5 , the assumption being that this phosphorus is present in the protein complex associated with calcium in the same proportions as in calcium phosphate. From these figures it may be calculated that 142 g. of P_2O_5 (the quantity contained in a gram molecule of calcium phosphate) are contained in 3480 g. of caseinogen. The remarkable approximation of the two numbers giving (1) the least molecular weight of acidic caseinogen on the assumption that an atom of phosphorus is contained in the molecule (3375) with (2) the quantity of caseinogen complex which contains a gram molecule of calcium phosphate (3480 g.) affords strong evidence in favour of the assumption made above, i.e. that caseinogen contains phosphorus in two forms (a) organically combined in the protein molecule, identical with the phosphorus in acidic caseinogen (0.021 g. P_2O_5 per gram of caseinogen) and (b) contained in caseinogen complex in association with calcium as $Ca_3(PO_4)_2$ (0.406 g. P_2O_5 per gram of caseinogen). This hypothesis receives further confirmation from a consideration of the quantity of acetic acid required to precipitate acidic caseinogen from caseinogen.

(e) *The precipitation of caseinogen by acetic acid.* On the addition of acid to a solution of caseinogen no precipitate is produced until a definite

quantity of acid has been added and then the whole of the acidic caseinogen comes out of solution. The following figures illustrate this statement.

Caseinogen solution	H ₂ O	Acetic acid (0.1 N)	
1 cc.	8.9 cc.	0.1 cc.	no pp.
	8.8 „	0.2 „	„
	8.7 „	0.3 „	„
	8.6 „	0.4 „	„
	8.575 cc.	0.425 cc.	trace pp.
	8.55 „	0.45 „	complete pp.

This precipitation will be considered more fully later. At present it may be noted that a definite quantity of acid precipitates a definite quantity of caseinogen, and that these relations are almost independent of temperature or dilution. In the above experiment the quantity of caseinogen contained in the solution was 2.68 g. per 100 cc. Therefore 0.45 cc. acetic acid (0.1 N) precipitated 0.0268 g. caseinogen, or in ordinary terminology, a gram molecule of acetic acid precipitated 583 g. of caseinogen from solution as acidic caseinogen. Now six molecules of acetic acid are equivalent to one molecule of calcium phosphate $\text{Ca}_3(\text{PO}_4)_2$. Therefore an amount of acetic acid which is equivalent to a gram molecule of calcium phosphate precipitates 3498 g. of caseinogen from solution as acidic caseinogen. The interest of this number lies in the fact that the quantity of caseinogen which contains a gram molecule of calcium phosphate is 3480 g.

Discussion of results.

The above results may be stated shortly as follows:

I. On the assumption that one unit of acidic caseinogen contains one atom of phosphorus the weight of acidic caseinogen containing one gram atom of phosphorus is 3375 g.

II. On the additional assumption that the phosphorus of caseinogen is contained in two forms (i) as organic phosphorus equal in quantity to that contained in acidic caseinogen, (ii) as inorganic phosphorus being contained in the protein complex as calcium phosphate we find (a) that the quantity of calcium and inorganic phosphorus contained in caseinogen bears the same relation to one another as is expressed in the formula $\text{Ca}_3(\text{PO}_4)_2$ and (b) that the amount of caseinogen which contains one gram molecule of calcium phosphate is 3480 g.

III. The amount of caseinogen precipitated by six gram molecules of acetic acid, i.e. a quantity equivalent to a gram molecule of calcium phosphate, is 3489 g.

The approximation of the above numbers—the quantity of acidic caseinogen containing a gram atom of phosphorus (3375 g.); the quantity of caseinogen containing a gram molecule of calcium phosphate (3480 g.); and the quantity of caseinogen precipitated by six gram molecules of acetic acid (3489 g.)—offers strong evidence in favour of the assumptions made and justifies the conclusions that,

(1) a unit of acidic caseinogen containing one atom of phosphorus has a weight of approximately 3500;

(2) caseinogen consists of a complex of protein having the same weight as the acidic caseinogen unit associated with one molecule of calcium phosphate;

(3) the precipitation of acidic caseinogen from caseinogen by acetic acid involves the reaction of the added acid with equivalent quantities of calcium phosphate, one molecule of calcium phosphate being replaced by six molecules of acetic acid.

The following tentative hypothesis is put forward as to the constitution of the caseinogen complex.

The protein contained in the complex may be expressed by the formula

of which the radicle (X) has acidic properties, possibly due to the phosphorus contained in it. The acidic radicle (X) reacts with the $Ca_3(PO_4)_2$, forming $X Ca$, whilst the liberated acid calcium phosphate combines with the amino group, the resultant complex having the formula

The experimental evidence in favour of this decomposition of $Ca_3(PO_4)_2$ into $Ca(OH)_2$ and $Ca_2H_2(PO_4)_2$ is derived from the work of Cameron and Seidell [1904] who showed that tricalcium phosphate and monocalcium phosphate are considerably decomposed by water but that dicalcium phosphate is relatively stable in this medium.

SUMMARY.

(1) Caseinogen may be prepared from milk by alcohol precipitation. The protein forms a milk white solution in water possessing all the properties of caseinogen contained in milk.

(2) Comparative analyses of caseinogen and acidic caseinogen indicate that caseinogen is composed of a complex of protein and calcium phosphate,

approximately 3500 g. of caseinogen containing a gram molecular weight of tricalcium phosphate.

(3) The amount of phosphorus contained in acidic caseinogen shows that a gram atom of phosphorus is contained in approximately 3400 g. of acidic caseinogen.

(4) Six gram molecules of acetic acid (a quantity equivalent to one gram molecule of calcium phosphate) precipitate acidic caseinogen from approximately 3500 g. of caseinogen.

(5) These relations suggest that the protein unit of acidic caseinogen has a weight of approximately 3400 g.; that caseinogen consists of a complex containing one unit of protein and a molecule of tricalcium phosphate; and that the precipitation of acidic caseinogen from caseinogen by acetic acid may be expressed thus:

The following relation of the protein to the calcium phosphate in caseinogen has been suggested:

where the group (X) has feeble acidic properties.

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