THE EFFECT OF RENNIN UPON CASEIN.

I. The Solubility of Paracasein in Sodium Hydroxide.

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(Accepted for publication, May 13, 1927.)

I.

INTRODUCTION.

When small amounts of rennin, or of any proteolytic enzyme, are added to milk a clot appears. In the course of the clotting a modification occurs in the casein of milk, resulting in the production of a protein that has been called paracasein.[1]

The clotting of milk has been the subject of many investigations, and the explanations of the phenomenon that have been proposed may be classified, on the whole, into two groups. The first of these assumes that the clotting of milk is essentially an incipient proteolysis. The second interprets the reaction in terms of colloidal chemistry and assumes that no chemical modification has taken place, paracasein being only a physical modification of casein. According to this view, the change leading to the clotting of milk involves some colloidal properties of the casein.

Among the exponents of the theories falling under the first group was Hammarsten. As early as 1875 Hammarsten attempted to explain the action of rennin in terms of proteolysis, the casein being split into two parts: one, paracasein, and the other a whey albumin (1) Very similar views were held by Schmidt-Nielson (2) and by Slowtzoff (3). Van Herwerden advanced a modified theory. According to this, the resulting paracasein is made up of two substances, Paracasein *B* and Paracasein *C*. The latter (unlike Paracasein *B*) is not precipitated by acetic acid, and requires more concentrated $(NH_4)_2SO_4$ for its precipitation. It is soluble in

[1] Casein for paracasein, and caseinogen for casein, according to the English classification.

water and gives a strong biuret reaction. After long action a proteose is formed which leads to the further decomposition of the molecule (4).

The analytical identity of casein and paracasein has been the subject of several investigations. Köster (5), Rose and Schulze (6), Raudnitz (7), L. L. Van Slyke and Bosworth (8) and Bleyer and Seidl (9) analyzed paracasein. Harden and Macallum (10) found that the "conversion of caseinogen into casein by enzyme action is accompanied by the cleavage of N, P and Ca. Rennin action produces no soluble N or P. Trypsin splits off both soluble N and P. . . . . The cleavage products are specific for each enzyme and it is to this difference of enzyme action that the variation in behavior of the resulting casein is to be ascribed."

TABLE I.

The Elementary Composition of Paracasein.

Per cent of the element						Investigator
C	H	N	S	P	Ash	
52.79	6.98	15.84				Köster (1881)
53.94	7.14	15.14	1.01			Rose and Schulze (1885)
				0.66–0.84		Raudnitz (1903)
				0.85–0.87		Kikkoji (1909)
53.50	7.26	15.80	0.87	0.83	0.61	Van Slyke and Bosworth (1913)
53.50	7.26	15.80	0.72	0.71	0.07	Bosworth (1914)
53.47	7.19	15.78	0.72	0.71	0.09	" "
		15.64	0.81	0.83	0.1	Bleyer and Seidl (1922)
		15.70	0.76	0.80	0.085	" " " "
		15.65	0.78	0.79	0.09	" " " "
		15.60				" " " "
		15.61				" " " "
Average....53.45	7.17	15.65	0.81	0.78		
Casein.....53.5–	7.13–	15.62–	0.72–	0.71–		
52.7	6.81	15.80	1.01	0.88		

Geake (11) investigated the elementary compositions of casein and paracasein and found them much alike.

Finally, Bosworth (12), using purified paracasein, found that the composition of paracasein was the same irrespective of the enzyme used to produce it. According to him, casein and paracasein have the same percentage composition. The results of these analyses are given in Table I. On the whole, paracasein does not differ much in elementary composition from casein.

We shall express our results in terms of nitrogen. In some of the calculations, however, it will be necessary to convert these values to

terms of gm. of paracasein. In such calculations we shall use the average value for nitrogen obtained from Table I.

Whether a proteose is split off from the casein molecule by rennin has been a subject of much controversy. For this there seems to have been a twofold reason. Not all clots formed by rennin are alike. Their abundance and physical properties largely depend upon the Ca and H ion concentration of the milk. It follows that when the coagulation occurs, soluble nitrogen can be occluded and absorbed to different extents. Rennin preparations usually contain some proteolytic enzyme, and it has often been contended that rennin is itself a proteolytic enzyme. Therefore, if the proteolytic enzyme is not inactivated in the course of the coagulation by the products of the reaction, it will afterwards produce soluble nitrogen, and even P or S, depending upon the manner in which the complex protein molecule is split.

The study of the nature of the reaction between casein and rennin has been most successfully investigated by the study of the intermediary product of the reaction—the paracasein isolated from milk and subsequently purified. Van Slyke and Bosworth (13), using their methods for the purification of casein and for the purification of paracasein, found that the amount of base just necessary to hold paracasein in solution was almost exactly twice that necessary to dissolve casein. They concluded that:

"the molecule of calcium caseinate containing four equivalents of base is split by rennin into two molecules of paracaseinate, each containing two equivalents of base. Such a paracaseinate is soluble in water, but insoluble in the presence of more than a trace of a soluble calcium salt. A molecule of calcium caseinate containing two equivalents of base is split by rennin into two molecules of paracaseinate, each containing one equivalent of base. Such a paracaseinate is insoluble in water."

Rennin therefore is not regarded by these investigators, strictly speaking, as a coagulating enzyme. Rather they consider the coagulation as a secondary effect, the result of a change in solubility. Further, they believe the action of rennin to be the first step in the proteolysis of casein (14).

Among the exponents of a purely physical point of view of the clotting of milk, Mellanby (15) must be mentioned. He supposed that the enzyme was adsorbed by the casein, thus forming the clot. Alex-

ander (16) suggested that the casein in milk is protected by the lactalbumin; and that the enzyme destroys the lactalbumin, and thus permits the coalescence of the caseinogen particles. Schryver (17), after an extensive investigation of the solubility of casein subjected to various treatments, concluded that in milk:

"the materials necessary for the clot formation pre-exist, but that aggregation formation 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."

Recently, Wright (18) investigated the racemization curves of casein and paracasein and found them identical. From this fact he concluded that

"rennet does not cause any proteolytic cleavage of the caseinogen molecule, but that coagulation is due to an alteration of the colloid state of the caseinogen by which precipitation in the presence of bivalent metal ions is facilitated."

The present investigation was undertaken on the basis of the simplest chemical hypotheses. In order to know the nature of a reaction, one must know the chemical properties, not only of the reactants, but also of the products. The reactant, casein, is well known because of the extensive investigations of Lacqueur and Sackur, T. B. Robertson, L. L. Van Slyke, E. J. Cohn and their respective coworkers. We propose therefore to study paracasein: its physicochemical properties and behavior. In this we hope to identify paracasein as a chemical substance, in the belief that a more complete knowledge of the reactants and of the end-products of reactions promoted by enzymes may give more reliable information regarding their nature, and perhaps also their mechanism.

II.

The Preparation of Paracasein.

Six different paracasein preparations have been used in this investigation. Of these the protein was in five cases precipitated from milk by active preparations of rennin, and in one by pepsin. The methods used for the purification of the paracasein were much like those already described by L. L. Van Slyke and Baker (19). A modification of their method (20) yielded reproducible results with casein and

with paracasein preparations. Since in the latter case the first precipitation was affected by the enzyme, it was in some cases unnecessary to add any acid. In this only did the method of purification of casein differ from that of paracasein. In one of the paracasein preparations we used a large excess of alkali, in order to study its effect on the protein. No appreciable differences were found between preparations treated with an amount of alkali just necessary to dissolve them and a preparation in which an excess of alkali had been used.

Paracasein I.—An active rennin powder prepared by the Digestive Ferments Company was used in the preparation of Paracasein I. To 7 liters of skimmed milk was added 0.001 gm. of the powder. The milk was then kept for 24 hours at 25°C. under an excess of toluene. After the separation of the clot the precipitate was washed seven times with four times its volume of distilled water. The precipitate was brought into intimate contact with the successive wash waters by means of a motor-driven, glass, screw-shaped stirrer. On each washing the reaction of the the clot appeared to be more acid, until by the seventh washing the pH had become 4.6. This reaction is the optimum for the precipitation of casein (21). The change of the reaction of the precipitate can most easily be accounted for in terms of the Donnan equilibrium; for, since both calcium paracaseinate and uncombined paracasein are relatively insoluble, it may be conceived that transformation of the former into the latter takes place by the diffusion of calcium ions from the casein clot, and their replacement by hydrogen ions. This case is thus comparable with the Donnan theory of hydrolysis through a membrane. It is of interest to note that this phenomenon did not take place with all of our preparations.

After the clot was washed, the paracasein was dissolved by the addition of 0.1 N sodium hydroxide. The reaction of the resulting solution was found to be approximately pH 7.0. The solution was passed through a Sharples centrifuge, and then filtered through filter paper pulp. The paracasein was reprecipitated by means of 0.1 N hydrochloric acid, delivered very slowly from a capillary tip extending well into the solution. The solution was continuously and rapidly stirred. The acid was added until the pH was 4.6, which was found to be the point at which paracasein flocculates the best. Finally, the precipitate was washed with water, redissolved by sodium hydroxide, reprecipitated by hydrochloric acid and again washed until chloride-free. The whole preparation was carried on in the manner that has already been described in detail for casein (20).

Paracasein II.—Pepsin powder prepared by the Parke, Davis and Company was used in the preparation of Paracasein II. To 4 liters of milk was added 0.002 gm. of the powder. The subsequent washing and purification was carried on in the same way as for Paracasein I.

Paracasein III.—Active rennin powder prepared by the Digestive Ferments Company was used in the preparation of Paracasein III. To 3½ liters of milk was added 0.005 gm. of the powder. The clot was washed four times as before, and enough 0.1 N sodium hydroxide was added to bring the solution to pH 9.0. The subsequent purification was carried on in the same way as in the cases of the Paracaseins I and II.

Paracasein IV.—3 liters of milk were warmed to 32°C. Immediately 0.036 gm. of Hansen's salt-free active rennin powder was added to it. The milk was vigorously stirred. After an elapse of 1½ hours, the milk coagulated. It was immediately cooled down to 5°C., and extensively washed with water.

Otherwise the procedures of purification were the same as in Preparations I, II and III.

Paracasein V.—In this preparation, we used the Morgenroth method of coagulation of milk. It is based on the following observation: Milk to which rennin is added and which is kept at a low temperature does not coagulate, although the transformation of casein into paracasein presumably takes place. This milk can be coagulated after a short exposure to high temperature. The usual explanation of this phenomenon is that paracasein, even in the presence of salts, does not coagulate at a low temperature. The coagulation may be brought about by warming the milk.

3 liters of milk were cooled to about 5°C., and 0.01 gm. of Hansen's salt-free rennin preparation was added to it. The milk was placed in a cold room at 5°C., and left undisturbed for 20 hours. No coagulation took place at that temperature. The milk was then warmed to 35°C., which brought about its coagulation in a time slightly less than 30 minutes. After the coagulation took place the milk was cooled to 5°C., and purified by the ways described.

Paracasein VI.—To 3 liters of milk we added, in this preparation, 0.03 gm. of considerably weakened Hansen's salt-free rennin preparation. After an elapse of about 12 hours at about 18°C. the milk coagulated.

The subsequent purification of this preparation of paracasein was carried on in the usual way.

Since, in the preparations of paracasein, an enzyme was added to the systems, they were no longer comparable to systems containing only casein. If, however, the enzyme added were wholly inactivated during the course of the purification, the investigation of paracasein would become comparable with that of casein.

Our pepsin preparation had roughly twice as much enzyme as the rennin preparations, both in respect to coagulative and proteolytic activity. Therefore, Paracasein II had about ten times as much enzyme as Paracasein I. The same relation is true of Paracaseins III and I. But, Paracasein III was also brought to an alkaline reaction in the course of its precipitation, to pH 9.0. It is well known that both pepsin and rennin are very sensitive to alkali, and experiments carried out in this laboratory upon the latter confirm the observation that this enzyme is quickly inactivated even at neutral reactions. We conclude from this that

after purification Paracasein III should contain less enzyme than Paracasein II.

If the property of prepared paracasein were dependent upon the proteolysis produced by rennin or pepsin added, we should expect that Paracasein II, when equilibrated with a certain amount of sodium hydroxide, would produce much more soluble nitrogen than, for example, Paracasein I or III. This was found not to be the case. Preparations of paracaseins differed but slightly from each other, and their solubilities were practically independent of time.

As we shall see from one of the next sections, the solubility of paracasein can be measured both at low and at high temperature. If the solubility is due, even in part, to a proteolytic enzyme, the rise in temperature would increase the solubility. On the contrary, the temperature coefficient of the binding capacity of paracasein with NaOH does not differ at all from the corresponding temperature coefficient of casein.

Therefore, we are inclined to think that in the course of the purification of paracasein the enzyme added was wholly inactivated, and did not interfere appreciably with subsequent measurements.

III.

The Solubility of Paracasein in Water.

Paracasein, purified to the same extent as casein and chloride-free, still contains large amounts of caseose which appear in the solution, after a thorough washing of the suspension. The amount of soluble nitrogen varied slightly from preparation to preparation, and the pH approached 6.5, presumably as a result of the presence of these substances. If we assume that paracasein is soluble in water, the nitrogen found in the filtrate may be represented as being composed of (1) nitrogen arising from the solution of paracasein in water and (2) nitrogen arising from any other soluble substance present in our system. In order to simplify our reasoning, let us consider first a simpler chemical case. Let us assume that we have in a liter of water a large excess of barium sulfate, together with some magnesium sulfate and sodium sulfate, and that we wish to measure the solubility of barium sulfate in water. Let our method for the estimation of barium sulfate be limited

to the measurement of the SO_4 radical only. Should one equilibrate such a mixture and then filter some of it and estimate the gm. of SO_4 present in the filtrate, values for the solubility of barium sulfate, greater than the theoretical solubility, would be obtained. The magnitude of this increment will of course depend upon the amount of more soluble sulfate salts present. An attempt, however, to wash such a system systematically, will result in values ranging closer and closer to the value of the solubility in water of the least soluble substance, barium sulfate in our case. Several criteria for the value of the solubility of barium sulfate in water might be applied. First, it should be independent of the way in which barium sulfate was prepared; in the second place, its solubility should be independent of the length of time the solvent is equilibrated with the saturating body, which should finally dissolve in successive fractions of the same solvent to the same characteritic extent, since a chemically pure substance has always the same physicochemical properties.

The situation with paracasein was very like that with the inorganic analog we have just considered. In our case paracasein, a substance of unknown solubility, was obtained with a mixture of other nitrogen-yielding substances, also of unknown solubility. The method used to obtain a pure substance from this mixture was identical with that described for the purification of barium sulfate.

The experimental procedure was as follows: Samples of Paracaseins I, II and III were placed in three Pyrex bottles. They were diluted with water to about ten times the volumes of the precipitates. The suspensions were stirred by means of a motor-driven, glass, screw-shaped stirrer at about 7°C. The stirring did not change appreciably the state of subdivision of the paracasein precipitate. Only a few hours were required to bring the suspension to the state of equilibrium with the watery phase. The time of stirring was, however, extended to 48 hours and often to more. After each stirring the supernatant liquid was decanted and part of it filtered through a No. 42 Whatman filter. The nitrogens were determined on 50 cc. aliquots by the Kjeldahl method. On the same filtrate a colorimetric pH measurement was taken. Then the bottles were refilled with cold water, and the operation repeated. The results of the experiment are tabulated in Table II.

The results of the experiment show a constancy of the solubility of paracasein in water, when freed from hydrolytic products. Only one measurement, namely, the solubility of Paracasein III on the seventh washing, diverges by a value greater than the experimental error.

We have reason to believe that this preparation became contaminated at this stage of washing. It was probably for this reason that the solubility of this preparation was slightly higher.

There is one more bit of interesting evidence that the system of paracasein and hydrolytic products is very similar to the system of our inorganic analog. Paracasein II, after the first and the third washings (Table II), was stirred for 5 and 10 hours, respectively, and then for 48 and 24 hours. The amount of nitrogen in the liquid phase remained constant. This evidently indicated that the soluble nitrogen

TABLE II.

The Solubility and the Hydrogen Ion Activity of Paracasein in Water.

Time of stirring	No. of washings	Solubility: mg. N in 25 cc. Paracasein preparation			pH Paracasein preparation		
		I	II	III	I	II	III
hrs.							
5	1		1.37			6.5	
53	1		1.37			6.5	
10	2		0.77				
10	3		0.48			6.4	
34	3		0.47				
12	4	0.35	0.33	0.41	6.4	6.4	6.4
48	5	0.14	0.16	0.17	6.4	5.25	6.2
48	6	0.14	0.15	0.20	5.25	5.25	5.25
72	7	0.15	0.17	0.25	5.1	5.2	5.25
48	8		0.15				
144	8	0.16					

did not arise from the activity of a proteolytic enzyme, since then, with time, the amount of protein hydrolyzed should increase. This was not the case. Our systen was a mixture of two or more substances differing in their solubility in water.

The average of all the determinations from the fifth down to the eighth washing gave the value of 0.17 ± 0.03 mg. of nitrogen in 25 cc. The solubility of paracasein per 1000 gm. of water is therefore equal to 6.8 ± 1.2 mg. of nitrogen, at about 7°C.

The pH of a purified paracasein is most probably 5.2. It is more alkaline than the pH at which paracasein is best precipitated, which is in the neighborhood of pH 4.6.

In an investigation of the effect of temperature upon the solubility of casein in water (22), we found that this protein dissolves to an extent of 7.0 ± 1.0 mg. protein N in 1000 gm. of water at 5°C. Comparing this solubility with the one obtained for paracasein, it is evident

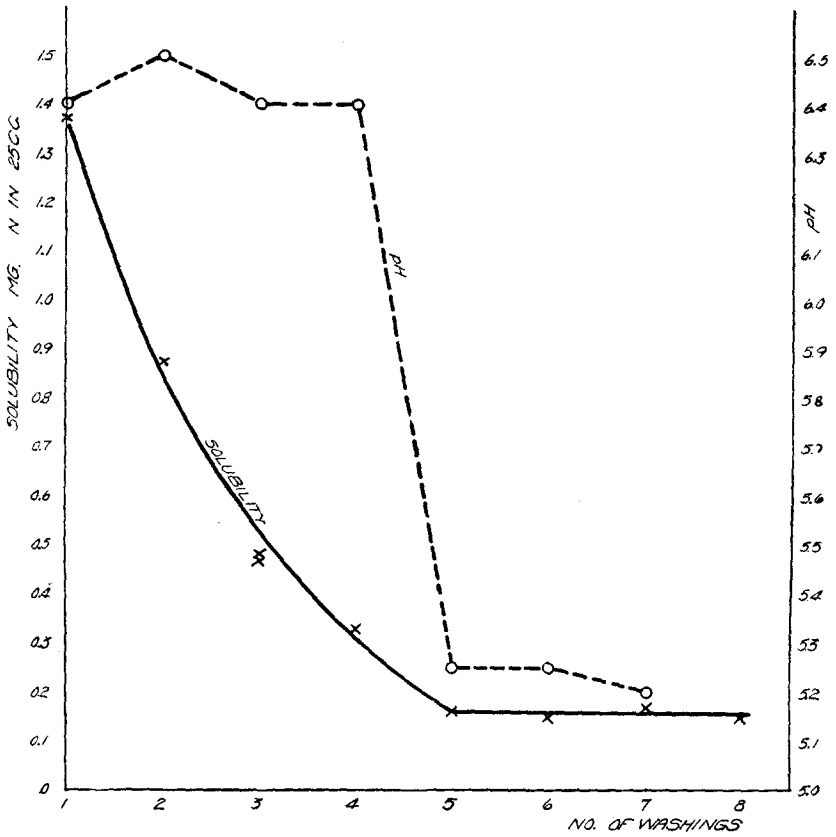

FIG. 1. The purification of paracasein from hydrolytic products and its solubility in water in an uncombined state.

that the solubilities of these two proteins are identical at practically the same temperature. We may conclude that paracasein, at low temperature, dissolves in water approximately to the same extent as casein.

IV.

The Solubility of Paracasein in Sodium Hydroxide.

Similarly to casein, when sodium hydroxide is added to paracasein, this protein forms a sodium compound which is soluble in water. By

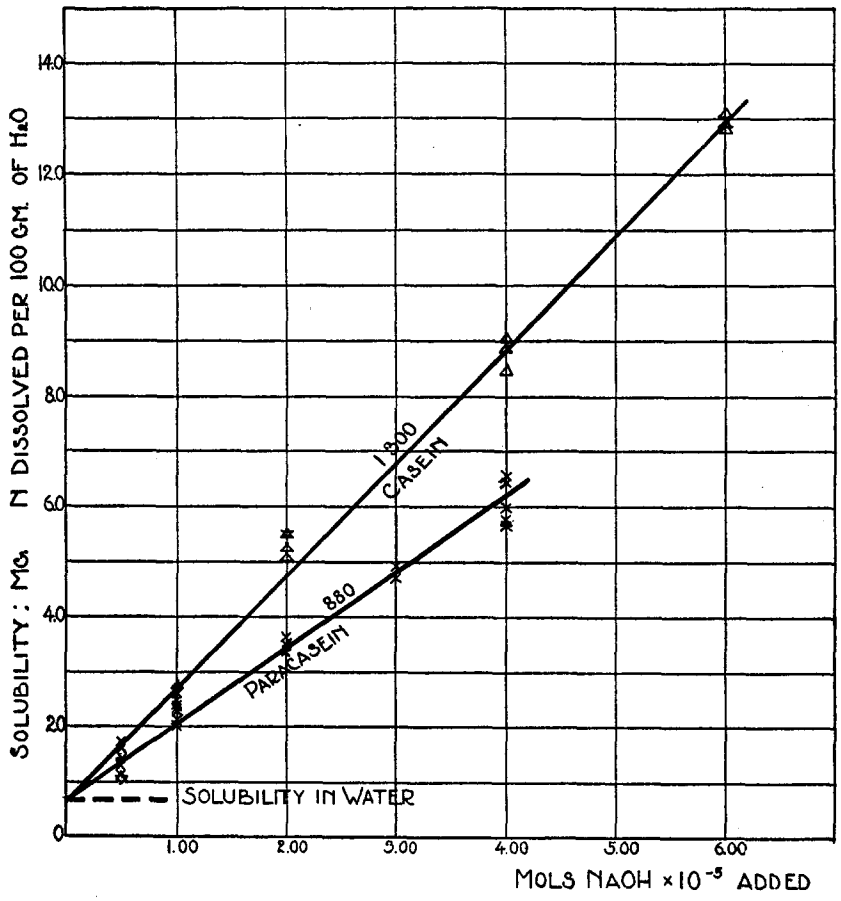

FIG. 2. The solubility of paracasein in NaOH at 5°C. as compared with the solubility of casein at the same temperature (22).

estimating the amount of paracasein dissolved by a given amount of sodium hydroxide, we can measure the combining capacity of this protein as an acid, provided all the base is bound by the protein in solution.

Before undertaking the actual measurements of the solubility of the protein, it seems to be advisable to determine the time in which this system will come to equilibrium. A sufficient criterion for this state for an isothermal, isopiestic system is the independence from time of the amount of protein dissolved by a given amount of base.

In order to elucidate this problem, we undertook the following experiment. Small amounts of Paracaseins II and III, in suspension, used in the experiments on solubility of these proteins in water, were pipetted out into 100 cc. volumetric flasks. To these flasks were then added small amounts of sodium hydroxide and carbon dioxide-free water until the volume of the solution in each reached the 100 cc. mark. The flasks were then placed in a shaking machine and equili-

TABLE III.

The Solubility of Paracasein in NaOH as a Function of Time.
Temperature: 5.0° ± 0.5°C.

Paracasein preparation	NaOH added: mols × 10^{-5}	Solubility: mg. N in 20 cc. of filtrate after an elapse of		
		1 hr.	3 hrs.	6 hrs.
(1)	(2)	(3)	(4)	(5)
II	1.00	0.52	0.52	0.54
III	0.50	0.23	0.23	0.24

brated for various periods of time at about 5°C. The contents of the flasks were then filtered through No. 42 Whatman paper filters and the filtrates analyzed for nitrogen by the Kjeldahl method.

The method used in this investigation was very similar to the one described by E. J. Cohn and Hendry (20) in their investigation upon the solubility of casein. The results of this experiment are given in Table III. They indicate, on the whole, that the protein dissolves readily in sodium hydroxide. The solubility of this protein is practically independent of time.

The investigation of the solubility of paracaseins in sodium hydroxide was then undertaken. The method for the determination of soluble nitrogen was identical to that one already described. The time of equilibration was varied from 2 to 24 hours. The temperature at which the experiments were carried out was 5.0° ± 0.5° C. and 23.0° ± 2°C.

The amount of the saturating body was varied within large limits without any appreciable effect upon the solubility of paracasein.

TABLE IV.

The Solubility of Paracasein in Sodium Hydroxide at 5.0° ± 0.5°C.

NaOH added: mols × 10^{-5}	Experiment No.	Paracasein preparation		
		I	II	III
		Mg. N dissolved per 100 gm. of water		
(1)	(2)	(3)	(4)	(5)
0.50	4 5 1	1.4 1.4	1.6 1.7	1.1 1.3
1.00	2 1 2	2.4 2.3	2.6 2.6	2.0 2.1
	4 2	2.3 2.4	2.6 2.7	
	3		2.4 2.4	
	5		2.4 2.6	
2.00	3 2 2	3.4 3.5	3.5 3.6	3.5
	4 5	3.4	3.4 3.4	
3.00	5		4.7 4.9	
4.00	3 5	5.65 5.75	6.45 6.55	
	4	5.65 6.00		

Paracasein is much like casein in this respect: the solubility with sodium hydroxide is independent of the amount of the protein in the precipitate, and is solely determined by the amount of the base added.

The results of these investigations are recorded in Tables IV and V, and Fig. 2.

The solubility of paracasein at 5°C. in NaOH (Fig. 2), unlike its solubility in water, is distinctly different from that of casein. An equal amount of base carries less paracasein into solution than it does casein. About 810 gm. of paracasein are carried into solution by 1 mol of NaOH.

TABLE V.

The Solubility of Paracasein in Sodium Hydroxide at 23° ± 2°C.

Paracasein preparation (1)	Amount of NaOH added: mols × 10^{-5} (2)	Solubility: mg. N dissolved per 100 gm. of water (3)	Equivalent weight: gm. of paracasein (4)
I	2.00	3.9	
	6.00	12.9	1450
II	0.50	(1.8)	
	1.00	3.3	
	5.00	11.9	1400
II	2.00	4.9	
	6.00	13.5	1400
IV	2.00	3.65	
	6.00	13.2	1550
V	2.00	5.35	
	4.00	10.1	1500
Average.....	.....	.....	1450
VI	2.00	5.17	
	4.00	10.55	1700

We have repeated these measurements at a temperature of 23° ± 2°C. In these investigations we have included Paracasein Preparations I and II, as well as IV, V and VI.

The results of these measurements are recorded in Table V. As in the measurements at 5°C., paracasein is distinctly different from casein at 23° ± 2°C.: its average equivalent is 1450, while casein in the

corresponding range of temperature had the equivalent weight of 2100 ± 100 gm. (20).

The equivalent weight of Paracasein Preparation VI, which was

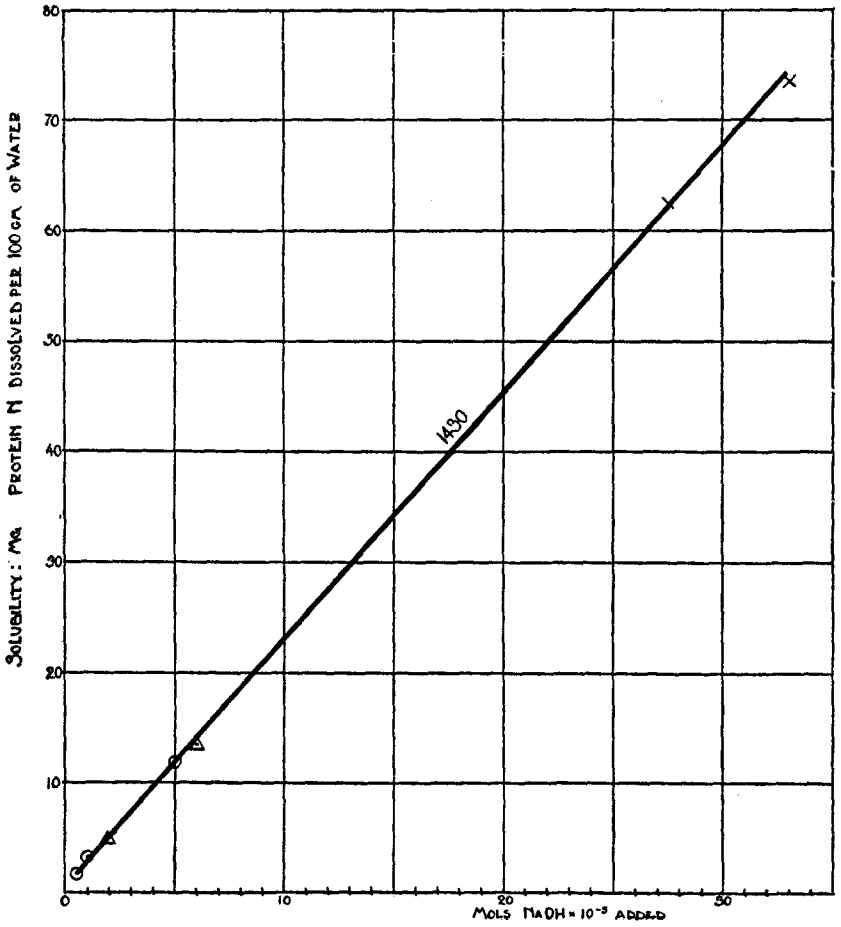

FIG. 3. The solubility of Paracasein Preparation II in small and large amounts of NaOH at 23° ± 2°C.

prepared by the use of a considerably weakened rennin preparation, is outside our experimental error. The equivalent weight of this preparation is about 1700 (Table V). We shall consider this paracasein preparation in the following communication.

In all of these investigations, we have confined ourselves to estimates of the solubility of paracasein with small amounts of sodium hydroxide. It is of interest whether paracasein, like casein (20) at 25°C., displays a constant base-combining capacity independent of the amount of base added. For this purpose we made solubility measurements upon Paracasein Preparation II with large amounts of NaOH, at 25°C. They are recorded in Fig. 3. The suspensions contained 86.0 mg. of paracasein N. With 32.9×10^{-5} mols of NaOH, 73.4 mg. N passed into solution. About 85 per cent of the protein was therefore dissolved. From the calculation of the equivalent weights of Fig. 3, it becomes evident that the solubility of Paracasein II is independent of the amount of base added. Like casein, this paracasein behaves toward NaOH at 25°C. as a homogeneous body; at least the 85 per cent of precipitate that dissolved appeared to be homogeneous.

V.

CONCLUSIONS.

The investigation was carried out upon a substance referred to as paracasein. It is not improbable that one can obtain from the clot produced by rennin, substances of diverse properties. Therefore, it is of importance to define our paracasein in rigorous terms. We shall refer to paracasein as a modification of casein produced in the clotting of milk by rennin or pepsin preparations, being redissolved several times by NaOH and possibly further acted upon by the enzyme; then washed at the maximum flocculation point until the product displays a constant solubility in water, independent of time and of the number of washings.

In studying the physicochemical properties of such a substance, it is of extreme importance to know whether the system contains any free enzymes.

The absence of the proteolytic enzyme from our parcasein preparation was concluded from the following observations: (1) The amount of enzyme originally added to the milk has no bearing upon the solubility of the product in water or NaOH. (2) The time factor has no effect upon the solubility of paracasein in water. (3) The solubility with sodium hydroxide is practically independent of time. (4) The

temperature coefficients of the solubilities of casein and paracasein in sodium hydroxide are identical (Table VI). To our mind, these criteria are sufficient to conclude that no active proteolytic enzyme was present in our purified paracasein preparations.

Paracasein dissolves in water to an extent of about 7.0 mg. of N per 1000 gm. of water at 7°C., which within the experimental error is identical with the solubility of casein at about the same temperature, since the properties of paracasein as an acid are distinctly different from those of casein, one may conclude that, in some cases, one can change the acid properties of a protein without affecting its solubility in water.

TABLE VI.

A Comparison of the Acid Properties of Paracasein and Casein.

Solubility range: mols NaOH × 10^{-5} (1)	Temperature, °C. (2)	Equivalent weight: gm. of protein		Ratio (3)/(4) (5)
		Casein (3)	Paracasein (4)	
0–6.00	5.0	1300		1.48
0–4.00	5.0		880	
Any	21–37	2100		1.45
"	21–25		1450	

As we have already pointed out, paracasein is distinctly different from casein in its capacity to bind base, both at 5° and at 23° ± 2°C.

At 5°C., paracasein, in most of the solubility regions investigated, dissolves at a rate of 810 gm. of the protein per 1 mol of NaOH. At the corresponding temperature and solubility range, casein dissolves to the extent of 1300 gm. per 1 mol of NaOH. At the temperature level of 21°–25°C., the corresponding figures are 1450 gm. for paracasein and 2100 gm. for casein.

This information is compiled in Table VI. In column (5) of this table is given the ratio of the equivalent weight of casein to the equivalent weight of paracasein. The relation seems to yield a constant, which indicates that the temperature coefficients of the solubility of paracasein and casein in NaOH are identical.

As the calculation of Table VI shows, paracasein is nearly 1.5 times

more acid than casein, the capacities of casein and paracasein to bind base standing to one another as the whole numbers 2 and 3 (22).

The fact that in using varying amounts of the enzyme one obtains practically identical parcaseins indicates that most of the paracaseins obtained in this investigation are final products of a reaction promoted or brought about by rennin or pepsin.

The information gathered in this research substantiates the conclusion reached by several investigators, that the transformation of casein into paracasein is of a chemical nature.

VI.

SUMMARY.

1. The preparation and purification of paracasein was described and certain criteria for the absence of free enzyme provided for.

2. The solubility of purified paracasein in water at low temperature was studied, and found practically identical with the solubility of casein.

3. The capacity of paracasein to bind base was investigated by means of its solubility in NaOH at 5° and at 23° ± 2°C., and found to be distinctly different from that of casein.

4. At these two temperature levels paracasein had a 1.5 greater capacity to bind base than casein. The equivalent combining weights of paracasein and casein were found to stand each to the other, approximately, as 2 to 3.

5. This relationship suggested that the temperature coefficients of the solubility of paracasein and casein in NaOH are identical.

6. This evidence indicates that paracasein is a modification of casein, distinguishable by physicochemical means.

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