XXXIV. THE ACTION OF RENNET AND OF HEAT ON MILK.

BY NORMAN CHARLES WRIGHT.

*From the Institute for Research in Animal Nutrition,
School of Agriculture, Cambridge.*

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I. THE ACTION OF RENNET ON MILK.

INTRODUCTION.

THE action of rennet on milk has been the subject of a number of investigations. Hammarsten [1872] was the first to show that the coagulation of milk by rennet was due to a soluble enzyme which, he supposed, acted on the caseinogen to produce an insoluble curd (Käse or Casein) and a soluble whey-protein (Molkeneiweiss).

Bosworth [1913] showed that the production of the latter was not due to the rennet action, and that the insoluble casein was the sole product of the reaction, a result confirmed by Harden and Macallum [1915]. Bosworth further confirmed the fact that the actual coagulation was not due to the rennet, but was a secondary effect due to the difference in solubility of the salts of casein.

Bosworth and Van Slyke [1913] in the course of extended investigations demonstrated that caseinogen and casein were analytically indistinguishable, a result also obtained by Geake [1914]. The former investigators claimed however that the salts of casein contained twice the amount of basic elements present in those of caseinogen, and on the basis of their analytical results they calculated the molecular weight of caseinogen to be 8888, and that of casein 4444. The general conclusion at which they arrived was that the rennet caused a proteolytic cleavage of the caseinogen molecule into two molecules of casein, which could then be coagulated by the addition of bivalent metal ions.

Other theories of rennet action have been put forward. Thus J. Mellanby [1912] supposed that the enzyme was adsorbed by the caseinogen and that it was the precipitation of the enzyme-caseinogen complex which caused the clot in milk. Alexander [1912] suggested that under normal conditions the caseinogen was protected by the lactalbumin of the milk, and that the enzyme, by destroying the lactalbumin, permitted the coalescence of the caseinogen particles. Schryver [1913] advanced a similar theory in which the coagulation was held to be due to the surface tension effects on the colloid constituents of the milk.

Up to the present time the only definite evidence of difference between caseinogen and casein is that based on the work of Bosworth and Van Slyke quoted above. The present investigation was commenced with the object of attempting to differentiate the two proteins by means of a study of their relative optical rotation and racemisation curves.

EXPERIMENTAL.

Woodman [1921] has shown that the identity of proteins may be determined with great accuracy by studying the curve of racemisation, namely the rate of racemisation of the protein in strong alkali as judged by the progressive diminution in optical rotation during incubation at 37°. Such a method is sufficiently sensitive to differentiate proteins as similar as serum albumin and lactalbumin, where other methods, biological and chemical, have failed to do so.

If the action of rennet is to cause a proteolytic cleavage of the caseinogen molecule, as suggested by Bosworth and van Slyke, we should expect to obtain different initial values of optical rotation, and possibly different rates of racemisation with caseinogen and its cleavage product. If on the other hand the action of rennet in producing a clot is simply a colloidal phenomenon, as suggested by other workers, then we should expect the racemisation curves of caseinogen and casein to be identical.

For the preparation of the proteins the method of Bosworth and Van Slyke was used, with the exception that 0.2 % NaOH was substituted for NH_4OH , in order to avoid possible high nitrogen values due to contamination with traces of ammonium salts. In order to determine whether alkali of this concentration effected any racemisation, a control preparation was made by the method of Baker and Van Slyke [1918], in which contact with alkali was avoided. The results (shown below) indicate that the dilute alkali does not cause appreciable racemisation.

Equal quantities of the caseinogen and casein were dissolved in alkali under standard conditions, the solutions incubated at 37°, and the optical rotations observed at intervals. The concentration of protein was checked by nitrogen determinations, and the specific rotation calculated. The readings of the first 24 hours were rendered difficult by the gradual formation of a slight suspension in the solution, which was found to consist of inorganic phosphate split off from the caseinogen by the action of alkali [Plimmer and Scott, 1908]. This suspension was completely precipitated by the end of the first day, and could be filtered off to give a clear solution, while the errors caused by the temporary turbidity were quite insufficient to invalidate any of the readings obtained.

Determinations were carried out on duplicate solutions and with several different preparations of caseinogen and casein and in all cases good agreement was obtained. The following is a summary of the results:

Table I. *Specific rotations (2 % in N/2 NaOH).*

Hours	Caseinogen (Baker and Van Slyke preparation)	Caseinogen (Bosworth and Van Slyke preparation)	Casein (Bosworth and Van Slyke preparation)
$\frac{1}{2}$	- 103.0	- 102.5	- 103.5
$2\frac{1}{2}$	—	- 89.5	- 88.5
6	- 80.0	- 79.0	- 79.0
9	—	- 74.5	- 74.5
24	- 66.0	- 64.5	- 64.5
50	- 55.0	- 55.0	- 55.0
100	- 42.5	- 42.5	- 42.0
169	- 38.0	- 39.0	- 38.0

DISCUSSION OF RESULTS.

It would appear from the results above that the two proteins, caseinogen and casein, must be considered chemically identical, a view which is in harmony with the fact that exhaustive analyses by several workers have shown them to be analytically indistinguishable.

The evidence against such a view is two-fold: (a) the difference in solubility of caseinogen after treatment with rennet, and (b) the difference in combining capacities of the two proteins. The first objection may be met by assuming that the rennet causes a change in the colloid condition of the caseinogen which facilitates precipitation in the presence of bivalent metal ions. With regard to the second objection, it seems doubtful whether the method used is in itself capable of sufficient accuracy to form a basis of differentiation, and in choosing between the conflicting evidence, one would rely rather on a physical method as being capable of the greater precision.

It has been urged in favour of the "cleavage" theory that the function of rennet is to cause the preliminary splitting of the large caseinogen molecule prior to more complete digestion by the ordinary proteolytic enzymes. Such a view may be met by the equally probable explanation that rennet coagulates the caseinogen of milk in order to allow the protein to be retained for a longer period in the sphere of action of the digestive juices.

II. THE EFFECT OF HEAT ON MILK.

INTRODUCTION.

The effect of heat on milk is here studied only in relation to its action on the caseinogen, a subject which has received comparatively little investigation.

Jensen and Plattner [1904] observed that the heating of milk to high temperatures caused the development of a brown coloration, and they supposed this to be due to the incipient breakdown of the caseinogen molecule. A number of investigators have observed that autoclaving milk at high temperatures (150°) causes actual coagulation of the caseinogen. Recently Hammarsten [1918] has claimed from his investigations to have shown that heating at 116° in an autoclave causes the splitting-off of an albumose-like body from the caseinogen.

From the nutritional side the subject has received more attention. Earlier work was directed to a study of the comparative digestibility of the raw and heated product. For a full discussion of the subject reference may be made to Lane-Claypon [1916] but it may be said that the general opinion tends to show that heated caseinogen is, on the whole, *more* digestible than unheated caseinogen. McCollum and Davis [1915], however, in experiments in which they heated caseinogen in an autoclave at 15 lbs. pressure, brought forward evidence that the heated product was no longer adequate to support growth, and claimed that "heat destroyed its biological value as a complete protein." Contrary evidence is forthcoming from Funk and Macallum [1916] and from Hogan [1917] both of whom base the loss of nutritive value on destruction of vitamins rather than alteration in the caseinogen molecule itself. Their opinion is supported by Hammarsten [1918] who concluded from his experiments that "inability of heated caseinogen to maintain growth of young rats cannot be ascribed to altered structure of constituent amino-acids."

The whole question is however in an unsatisfactory state, and it seemed that further investigation, particularly from the chemical standpoint was necessary.

Woodman's method of following the racemisation curve of a protein as a means of identification seems to offer excellent opportunity of detecting qualitatively whether any fundamental change takes place in the caseinogen molecule on the application of heat. If there is any alteration in, or displacement of, the amino-acid content, we should expect such a method to give a most sensitive indication of the change.

EXPERIMENTAL.

Fresh whole milk was taken and the cream removed by centrifuging. Equal quantities (1 litre) were treated separately as follows:

- (a) Unheated (control).
- (b) Pasteurised at 60° for half an hour.
- (c) Boiled under reflux condenser for half an hour.
- (d) Autoclaved at 120° for half an hour.

The various solutions were then precipitated with acetic acid and the caseinogen separated and purified by the method of Bosworth and Van Slyke. All the products were obtained finally as fine powders, (a) and (b) white, (c) slightly yellowish, and (d) light sand-coloured. The two former gave colourless solutions in NaOH, (c) slightly yellowish, but (d) gave a distinct reddish-brown solution which in high concentrations had a tendency to become gelatinous. The coloration could not be removed by water or any of the common solvents, or by dialysis.

Equal quantities of these four preparations were dissolved in standard alkali and their racemisation curves observed as in the previous experiments (see p. 246). The following is a summary of the results obtained:

Table II. *Effect of heat on caseinogen.*

Hours	Specific rotations (2 % in N/2 NaOH)			
	from raw milk	from pasteurised milk	from boiled milk	from autoclaved milk
2½	-89.5	-88.0	-92.0	-91.0
9	-74.5	-76.0	-74.0	—
24	-64.5	-65.0	-67.0	-67.5
50	-55.0	-55.0	-52.0	-56.5
100	-42.5	-44.0	-43.5	-42.0
169	-39.0	-38.0	-37.0	-37.0

DISCUSSION.

These results show clearly that, within the limits of the temperatures studied, heat effects no alteration in the constitution of the caseinogen molecule.

There remains to be explained the coloration and susceptibility to gelatinisation of the autoclaved product, and the increased digestibility as found by Hammarsten and other workers. The results obtained above indicate that alteration in physical state can be the only cause of the two latter phenomena. This fact is borne out strikingly by the experiments of Brenneman [1913] on the cause of increased digestibility. By using as a subject for his experiments a boy who could without discomfort regurgitate his food, he found that whereas with raw milk, the curds formed in the stomach were large and tough, with boiled milk they were soft and finer in character. Such an alteration in texture is explained on the basis of a difference in colloid condition of the raw and boiled caseinogen.

With regard to coloration, a control experiment was carried out in order to determine whether the caseinogen itself was the origin of the browning in autoclaved milk. A neutral solution of 4 % calcium caseinogenate (made by dissolving caseinogen in excess of CaCO_3 and filtering off excess of carbonate) was autoclaved for half an hour at 120° . There was no change in colour and the racemisation curve corresponded to those previously obtained.

Caseinogen from autoclaved calcium caseinogenate, 2 % in N/2 NaOH.

Hours	Specific rotation
2½	-90.0
9	-75.0
24	-65.0
50	-56.0
100	-43.0
169	-38.5

It follows therefore that the coloration obtained by autoclaving milk is due to the action of heat on some constituent other than the caseinogen, and that the apparently permanent coloration of caseinogen precipitated from such milk is due to the adsorption of pigment by the protein, and not to alteration in the caseinogen molecule itself.

The cause of coloration in autoclaved milk.

In order to determine which constituent of milk is responsible for the coloration of the autoclaved product, solutions of the following percentage composition were made up and autoclaved at 120°.

(a) Calcium caseinogenate, 4 %.

(b) Calcium caseinogenate, 4 %.

Lactose, 4 %.

(c) Calcium caseinogenate, 4 %.

Cream, 5 %.

(d) Lactose, 4 %.

At the end of half an hour, (a) remained white, (b) was dark brown, (c) slightly tinted, and (d) light yellowish brown. These experiments indicate that lactose is the precursor of the pigment in autoclaved milk. The slight coloration in (c) was probably due to traces of lactose in the cream, while the marked difference between (b) and (d) may show that the reaction is catalysed in the presence of a colloid substrate of calcium caseinogenate.

On acidifying (b), a precipitate was thrown down which adsorbed the pigment, giving on purification a light sand-coloured powder identical with that obtained from autoclaved milk.

SUMMARY.

(1) The action of rennet and of heat on solutions of caseinogen has been investigated by means of a study of the racemisation curves of the raw and treated protein.

(2) It is shown that caseinogen and casein have identical racemisation curves, evidence which supports the view that rennet does not cause any proteolytic cleavage of the caseinogen molecule but that coagulation is due to an alteration in the colloid state of the caseinogen by which precipitation in the presence of bivalent metal ions is facilitated.

(3) Within the limits of the temperature studied (up to 120° for half an hour) heat does not affect the constitution of the caseinogen molecule. Any difference in digestibility must be due to alteration in the physical (colloid) condition of the protein.

(4) The coloration of autoclaved milk is due to the caramelisation of the lactose, a reaction which appears to be catalysed by the presence of a colloid substrate of calcium caseinogenate.

The colour of caseinogen precipitated from autoclaved milk is due to adsorption of this pigment, and not to alteration in the caseinogen molecule itself.

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