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PROTEOLYSIS IN COWS' MILK PRESERVED BY MEANS OF FORMALDEHYDE.

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BABCOCK and Russell, in 1897, announced the discovery of galactase, a trypsin-like enzyme secreted by the milk glands, and attributed to it rather than to bacteria or bacterial enzymes the principal part in the proteolysis which takes place during the ripening of cheese. Since that time the digestion of milk proteids by galactase and by bacterial enzymes has been studied in considerable detail as regards its relation to cheese-making, especially by Babcock, Russell, and associates,[1] Van Slyke and Hart,[2] Freudenreich,[3] Jensen,[4] and Rogers.[5] Since in most of the experiments upon milk, ether or chloroform has been used to suppress bacterial action, the following observations upon milk preserved by means of formaldehyde are believed to offer some points of interest in connection with the more detailed studies of proteolysis which are being carried out elsewhere.

Samples of milk which had been treated with formaldehyde (about 1 : 1000) within two hours after milking, and which had been opened, mixed, and analyzed within the next few days, were subsequently kept in tightly stoppered bottles at laboratory temperature, usually exposed to diffused but never to direct sunlight. Since the original reason for thus preserving the samples was simply to provide for possible repetitions of the chemical analysis, no special precautions were observed regarding the exact amount of formaldehyde added or the time elapsing in each case before the final sealing of the sample. It has, however, been shown[6] that formaldehyde disappears only slowly when added to milk in such large proportions as were here used, and there is little doubt that each of the samples contained 0.07 to 0.10 per cent. of formaldehyde when finally set away, so that

[1] Reports of the Wis. Agr. Expt. Station, 1897-9.

[2] Bulletins 203, 219, 231, 233, 236 of the N. Y. State Agr. Expt. Station.

[3] Landw. Jahrb. Schweiz, 14, 49 (1900).

[4] Ibid., 14, 197 (1900).

[5] Bull. 62, Bureau of Animal Industry, U. S. Dept. Agr.

[6] Williams and Sherman : This Journal, 27, 1497 (1905).

any organisms or enzymes originally present or gaining access to the milk during the analysis[1] must have resisted the action of that amount of formaldehyde in order to have brought about the changes which were subsequently observed. Almost without exception such samples have remained entirely normal in appearance for about a year,[2] after which the milk begins to lose its opacity immediately below the cream layer and continues very gradually to show more and more distinct evidence of digestion. Some samples which have stood three to five years contain relatively very small clots or precipitates of curd, which still appear to be diminishing in amount.

During the summer of 1904 several of these samples were examined for bacteria by Professor R. A. Wardall, of the South Dakota Agricultural College, who was at the time studying in this laboratory. Liquefying cocci, forming yellow colonies, were found in most of the samples examined. White colonies of cocci and bacilli were also occasionally found. In some cases no development was found either on agar or in lactose bouillon. In all cases in which bacteria were found, they were few in number and developed so slowly that a routine examination might easily have resulted in the samples being pronounced practically sterile.

Thus while there is strong probability that bacterial action was quite thoroughly suppressed, it cannot be assumed that the digestion taking place in these samples was a true autolysis due simply to the proteolytic enzymes secreted with the milk, since absolute sterility was not attained in most cases, and especially since it appears from the work of Freudenreich[3] that bacterial remains and spores may yield proteolytic enzymes in the absence of vegetative forms, and from the observation of Van Slyke and Hart[4] that the udders of some cows always contain liquefying bacteria which appear to secrete proteolytic enzymes in the milk even before it is drawn. We cannot, therefore, expect to distinguish sharply between the action of galactase and of bacteria

[1] In one case duplicate samples about a year old were analyzed, one having been used for chemical analysis as described, while the other had received the same amount of formaldehyde at the beginning and had never been opened. The results were practically the same.

[2] During this time the greater part of the formaldehyde originally present will usually have disappeared.

[3] Loc. cit.

[4] Bull. 203, N. Y. State Agr. Expt. Station.

or bacterial enzymes unless as the result of comparative studies of the nature of the fermentations taking place under different conditions.

Although none of our samples was large enough to permit identification of the end-products, it was thought that a general idea of the character of the proteolysis could be obtained through the determination of the distribution of the total nitrogen among the principal groups of products at different stages of the digestion. The methods adopted for the separation and estimation of the nitrogen compounds are practically those of Van Slyke and Hart[1], and are therefore only very briefly outlined.

Total nitrogen, as well as the nitrogen in each of the fractions, was determined by the Kjeldahl-Gunning method.

Casein was precipitated by diluting 10 grams of milk with 90 cc. of water and acidulating at a temperature of 40° with 1.5 cc. of 10 per cent. acetic acid; the precipitate was filtered, washed, and its nitrogen determined.

For the determination of albumin and syntonin the filtrate from the precipitation of casein was very carefully neutralized by caustic alkali, using phenolphthalein as indicator, and heated to boiling until the precipitate settled, when it was filtered, washed and the precipitate treated for the determination of nitrogen.

The filtrate from the precipitation of albumin and syntonin was heated to 70°, acidulated with 1 cc. of 50 per cent. sulphuric acid, then saturated with zinc sulphate, filtered, and the precipitated proteoses washed with a saturated zinc sulphate solution and finally determined by the Kjeldahl-Gunning method.

The filtrate from the proteoses was saturated with bromine and the precipitate washed with bromine water and determined as "peptones by bromine."

For the determination of peptones and of amino and ammonium compounds two portions of the original sample of milk were treated separately with tannin and with phosphotungstic acid as follows: (1) To 40–50 grams milk in a 250 cc. flask, add 1 gram sodium chloride and 150 cc. of water, and then precipitate the proteids by adding a 12 per cent. solution of tannin, shaking after each addition and carefully avoiding any considerable

[1] Bull. 215, N. Y. State Agr. Expt. Station ; Amer. Ch. J. 29, 150 (1903).

excess of the reagent. Finally dilute to volume, filter, and determine nitrogen in 50 cc. of the clear filtrate. The nitrogen of the precipitate minus that of the casein, albumin, syntonin, and proteoses already determined is considered as belonging to "peptones by tannin." The nitrogen in the filtrate is taken as a measure of the "amino-compounds and ammonia by tannin."

(2) To 40–50 grams of milk in a 250 cc. flask add 150 cc. of water, 5 cc. concentrated sulphuric acid, and a 30 per cent. solution of phosphotungstic acid as long as a precipitate continues to be formed; then fill to the mark and complete the determination and the calculation of the results as in the case of the tannin precipitation.

On analyzing four samples of the same lot of milk eleven months old, two of which had stood at room temperature throughout, while two had been kept at 38° to 40° during the greater part of the last twelve days, no variation of results attributable to this difference of temperature could be found. It was therefore assumed that fluctuations of room temperature could have no appreciable effect upon the course of the proteolysis.[1] The average of these four analyses is taken as a basis for comparison in considering the nature of the changes which occur as digestion advances.

The accompanying table shows the principal results obtained.

The distribution of nitrogen found in the different samples indicates that the albumen was largely digested before the original amount of casein was appreciably reduced, but that subsequently, as digestion advanced and the caseïn diminished, the proportion of nitrogen existing as albumin and syntonin changed but little, the increase appearing first in the proteoses, then in the peptones and amino-compounds. In the last sample the proteoses and peptones had evidently been very largely broken down into amino-compounds, although small amounts of casein and acid albumin still remained. In general only a little more nitrogen was precipitated by phosphotungstic acid than by tannin, indicating that the precipitation of peptones by tannin must have been

[1] Jensen (loc. cit.) also found that milk containing formaldehyde digested about as rapidly at room temperature as at 35°, apparently because the stimulation of the galactase by the higher temperature is counterbalanced by the fact that formaldehyde affects that enzyme more injuriously in warm than in cold solution.

DISTRIBUTION OF NITROGEN IN SAMPLES OF MILK PRESERVED BY MEANS OF FORMALDEHYDE.

	Quadruplicate sample. 11 months old.	Sample No. 5.[1] 17 months old.	Sample No. 6. 26 months old.	Sample No. 7. 43 months old.	Sample No. 8. 28 months old.	Sample No. 9.[2] 37 months old.	
Lactose, per cent	4.25	4.37	4.46	4.46	4.68	4.76	
Estimated loss of lactose, per cent	0.31	...	0.39	0.38	0.18	0.15	
Acidity calculated as lactic, per cent	0.24	0.21	0.31	0.54	0.84	1.43	
Percentage of the total nitrogen, found as						A.	B.[2]
Casein	80.6	73.7	73.7	11.4	3.7	4.5	17.9
Albumin and syntonin	5.0	4.4	6.3	6.8	5.5	4.8	4.2
Proteoses	6.1	14.2	11.1	24.9	29.9	12.3	10.6
Peptones by bromine	0.6	0.9	2.3	10.1	10.7	...	...
Peptones by tannin	1.9	1.7	3.0	9.1	...	1.6	1.4
Peptones by phosphotungstic acid	2.2	1.9	5.2	10.7	...	...	...
Amino acids, etc., by tannin	6.4	5.9	5.9	47.8	50.2 (by dif.)	76.8	65.9
Amino acids, etc., by phosphotungstic acid	6.1	5.7	3.7	46.2		...	...

nearly, if not quite, complete and that only small amounts of diamino-acids, precipitable by phosphotungstic acid but not by tannin, could have been present in these samples.

Babcock, Russell, Vivian and Hastings[3] found a similar small difference between the tannin and phosphotungstic acid precipitates in milk digested by galactase, while in samples digested by bacterial enzymes the differences were much larger. Moreover, in our experiments relatively large amounts of proteoses were found at a rather advanced stage in the digestion, a result similar to that obtained by Babcock and associates in galactase proteolysis, but entirely different from that of bacterial digestion of milk. The proteolysis which had taken place in our samples,

[1] A commercial sample treated with formaldehyde when about one day old and not subsequently opened. All of the other samples had been treated as previously described.

[2] This sample contained a small lump of very tough curd resembling a corroded rubber stopper. Column A gives the results of analysis of the sample without this lump; in column B the figures are recalculated on the assumption that all of the nitrogen in the lump was in the form of casein. For most of the determinations upon this sample we are indebted to Mr. W. N. Berg.

[3] Ann. Report Wis. Agr. Expt. Station, 1899, p. 165.

therefore, agrees in essential features with that brought about by Babcock and Russell's galactase, although in view of the work of Van Slyke and Hart, Freudenreich, and Jensen, it appears probable that in both cases bacterial enzymes may also have taken part in the digestion.

Sample No. 8 preserved by the addition of 0.1 per cent. formaldehyde developed an acidity corresponding to 0.84 per cent. lactic acid in twenty-eight months, and during this time lost only 0.18 per cent. of milk-sugar, or less than 4 per cent. of the original amount, while 95 per cent. of the original casein had undergone digestion. In experiments made by A. W. Hahn and A. J. Mettler in this laboratory upon samples of milk preserved by the addition of 0.1 per cent. sodium fluoride, sodium salicylate, or hydrogen peroxide, the total acidity calculated as lactic acid has never equaled the amount of lactose destroyed, while up to the point at which 25 to 30 per cent. of the original lactose has disappeared, no marked digestion of casein has ever been noticed.

These analyses, therefore, in addition to showing the general nature of the very extensive proteolysis which had taken place, afford a striking illustration of the effect of an added antiseptic in determining the character of the fermentation which subsequently occurs in the milk.

EXTRACTION APPARATUS.

BY ALLEN ROGERS.

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THE author has obtained very excellent results by use of the herein-described apparatus in the analysis of such substances as tea, coffee, tannin and so on, where an aqueous infusion is necessary.

Two flasks *a* and *b* are employed and may be of any size desired. From the bottom of *b* a glass tube connects with *c* which contains the material to be extracted; at the end of *c* is a Bunsen valve. To charge the apparatus the clamp *d* is opened and water introduced through *e*; the clamp *d* is then closed, and suction applied at *f*, thus causing the liquid to pass over the material in *c*. The solution having passed from *b* to *a* the clamp *d* is again opened, and by blowing at *f* it is forced back into *b*. When a concentrated solution has been obtained in *a* it may be removed