XVI. ON THE PRESENCE OF AMYLASE IN MILK AND CHEESE.

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THERE has been little investigation regarding enzymes in raw milk, especially amylase. In 1883, according to the literature, Béchamp [1883] isolated from human milk an enzyme which liquefies starch and converts it into sugar, and named it galactozymase. He claimed that the enzyme was not present in cow's milk. His work was recognised by Bouchut [1885], Moro [1898], Biolchini and Luzzati [1902], Hippius [1905], and Spolverini [1902]; the last named found very little amylase in dog's, ass's and human milk and none in cow's and goat's milk.

Zeitscheck [1904] found evidence of the existence of slight amylolytic activity in all the samples of human, ass's, horse's, goat's, buffalo's and cow's milk examined by him. Spontagh [1905] also proved the presence of amylase in cow's and other milk which he examined.

Wohlgemuth and Strich [1910] were unable to obtain any evidence of the presence of amylase in cow's milk but found it in dog's, rabbit's and guinea-pig's milk, dog's milk containing most and guinea-pig's the least.

Koning [1907] recognised, on all occasions, evidence of amylase in cow's milk; thus he found that about 15–20 mg. of starch were decomposed by 100 cc. of milk in half-an-hour at 37°; and Giffhorn [1909] also found that 100 cc. of milk will decompose from 10–25 mg. of starch in one half-hour at 37°. Lane-Claypon [1913] carried out investigations upon the amylase content of sterile cow's milk collected by means of a collecting tube. Amylase was found in each experiment, but the amount present was so small that the 10 cc. of milk used were able to hydrolyse only from 0.001–0.002 g. of starch in three hours at 37°.

Regarding the activity of amylase in milk Koning found that the optimum temperature was 45° and that it was destroyed by heating the milk for 45 minutes at 68°, but Giffhorn found that the enzyme action was destroyed by heating at 65° for 30 minutes.

In regard to the source of this ferment in cow's milk there are some diversities of opinion among investigators.

According to Spolverini this ferment was obtained from cow's and goat's milk, which did not generally contain amylase, when the animals were fed with fodder containing a large quantity of starch, such as malt, and in such cases he was of opinion that a part of the amylase of milk may be derived from the udder and illustrated it by Dupony's experiment in which he proved that the amount of amylase was increased in the blood, liver and kidney by feeding with carbohydrates. But this opinion was denied by van der Velde and Landtscher [1909] who got negative results by the same experiment.

Koning showed that amylase was present in milk of animals with mastitis and that in normal milk the first and middle milks were richer than the strippings and also that certain bacteria were capable of producing amylase, but he believed that the amylolytic action obtained by him with cow's milk was not due to contamination by bacteria. Giffhorn also believed that amylase is increased considerably in milk by the pathological condition of the udders.

Wohlgemuth and Strich insisted that a part of the amylase of milk may be derived from the blood, but the milk generally contains at least 100 times as much amylase as the blood, the great part of this being a product of the activity of the mammary glands. Grimmer [1913] obtained evidence of its presence by his experiment on the gland tissue of animals of which the milk showed only little or no trace of amylase. Upon this experimental basis he suggested that amylase in milk was the product of the mammary gland.

As an application of the detection of the existence of amylase in milk, Koning carried out an experiment with the object of discovering whether milk has been boiled before being sold, and also stated that the presence of milk from a mastitis cow can be discovered by testing for this ferment. The former is generally used as a supplementary test to detect whether milk has been boiled or not, but the latter observation is not, at present, recognised as a reliable aid in the diagnosis of mastitis.

As to the degree to which starch is broken down by the amylase in milk, Moro [1902] (working with human milk) believed that some of it was converted into dextrin and maltose but that most of it remained in the stage of dextrin. The difficulty of estimating the terminal point of this reaction in milk is, however, very great, since it is almost impossible to detect the formation of minute quantities of maltose.

In short, at the present time nothing definite is known regarding the existence, origin and biological action of this enzyme in cow's milk. We also lack knowledge of any literature on amylase in cheese.

In the present investigation, as the first step, we examined raw milk for the existence and activity of amylase, and likewise the water-extract of fat-free cheese, determining in each case the power of liquefying potato- and also rice-starch, to see what difference if any exists in the effect of the amylase of raw milk and of cheese in liquefying these two starches.

Materials.

Milk and cheese.—The grade *A* raw certified milk and American Cheddar cheese used in the experiment were supplied by the Sheffield Farm Company in New York city.

Distilled water.—The distilled water which was used in the experiment was twice redistilled, first from alkaline permanganate solution into a reservoir whence it siphoned into a second flask from which it was again distilled. Phosphoric acid was added to this portion in order to retain any ammonia that might have come from the first distillation. Tin condensers were used and the final distillate was caught in "Nonsol" bottles, contact with the air being avoided as far as possible.

Substrates.—The substrates used in the experiment were eight samples, five of potato- and three of rice-starch, prepared by the following methods:

No. 1. Commercial potato-starch as purchased and further purified by repeated washing with distilled water.

No. 2. Potato-starch was prepared in the laboratory from mature August potatoes, care being taken to prevent contamination by organisms, dust, fumes, or water containing electrolytes.

No. 3. Potato-starch was prepared from potato-starch No. 2 by washing with 0.3 % NaOH.

No. 4. Lintner soluble potato-starch purchased from Merck and further purified with distilled water.

No. 5. Soluble potato-starch prepared from potato-starch No. 2 in the laboratory by Brown and Morris' method of treatment with dilute hydrochloric acid.

No. 6. Rice-starch prepared in the laboratory from the commercial product in the same way as potato-starch No. 2.

No. 7. Rice-starch prepared from rice-starch No. 6 by washing with 0.3 % NaOH.

No. 8. Soluble rice-starch prepared from starch No. 6 in the laboratory in the same way as the soluble potato-starch (No. 5).

NOTE:—For convenience, the different preparations of starch are designated by the following figures:

P.S. I = Starch No. 1	P.S. V = Starch No. 5
P.S. II = " " 2	R.S. I = " " 6
P.S. III = " " 3	R.S. II = " " 7
P.S. IV = " " 4	R.S. III = " " 8

Apparatus.

Thermostat.—The digestion of starch by the amylase was carried out in a bath which contained water kept at 40° by means of carbon filament lamps. The water in the bath was stirred constantly to ensure an even temperature throughout the experiment.

Glassware.—All glassware used in the experiment was treated to avoid any condition which could effect enzyme action.

Analysis of the starch.

The moisture, amylose and acidity of the various samples of starch were determined by means of the following methods:

Moisture. From 0.2 to 1.0 g. was dried in an air-oven at 100°–110° for four hours, allowed to cool in a desiccator over sulphuric acid for half-an-hour, and weighed.

Amylose. Amylose was determined by hydrolysing to glucose and estimating the glucose by Fehling's volumetric method.

Acidity. Acidity was determined by titration of the starch paste and solution with 0.001 *N* sodium hydroxide or sulphuric acid, using rosolic acid as indicator.

The results obtained are given in Table I.

Table I.

Substance	Moisture %	Amylose %	Acidity (cc. of <i>N</i> /1000 acid or alkali, per 1 g. amylose)
P.S. I ...	14.64	84.59	9.21 (H ₂ SO ₄)
P.S. II ...	13.79	85.88	7.19 "
P.S. III ...	16.65	89.29	14.22 "
P.S. IV ...	9.73	90.00	3.83 (NaOH)
P.S. V ...	13.84	85.55	2.44 (H ₂ SO ₄)
R.S. I ...	8.43	91.09	3.49 "
R.S. II ...	11.69	86.54	7.89 "
R.S. III ...	9.26	90.36	4.21 "

Preparation of potato- and rice-starch paste and solution.

The amount of starch equivalent to 1 g. of amylose was placed in an Erlenmeyer flask and 80 cc. distilled water added. The liquid was then heated slowly, with constant shaking, until it boiled, and the boiling was continued during three minutes. The flask was then cooled and the alkalinity or acidity of the starch was corrected by adding to each starch paste or solution sufficient *N*/1000 H₂SO₄ or *N*/1000 NaOH to make it exactly neutral, after which the volume was made up to 100 cc. with distilled water and the sample kept in an ice-box at 7°–8° until used.

The amylase activity of raw milk.

As soon as the milk sample reached the laboratory, it was well mixed and, after the estimation of its acidity with *N*/10 NaOH, was divided into two portions, one of which was kept raw, the other pasteurised by heating for 30 minutes at about 80°. The former was used for the enzyme examination, and the latter as control.

The following procedure based upon the method of Koning with slight modification was used.

Two series of test-tubes were very carefully cleaned and dried, placed on the test-tube-stand and the whole set in a bath of ice-water. Then known

quantities of starch paste or solution were carefully measured in increasing amounts, *e.g.* 0.001 cc., 0.002, 0.003, ..., into each of the tubes by means of a long 1 cc. pipette (divided into 1/100 cc.) reaching to the bottom of the test-tube, thus avoiding the lodging of any of the solution on the side of the tube.

10 cc. of raw or pasteurised milk, which had been kept in the ice-box at 7°–8°, were very accurately measured and introduced into each tube and agitated to ensure a perfect mixture.

The test-tube-stand was transferred from the ice-water to the thermostat and after various lengths of time was taken out and placed in the ice-water to stop the action. After a few minutes exactly 1 cc. of iodine solution (1 g. iodine, 2 g. potassium iodide + 300 cc. water) was added to each tube, and the contents thoroughly mixed. The tube in which the blue or violet colour had entirely disappeared, giving place either to red, orange or yellow, was noted and the power of the enzyme was calculated as the number of grams of starch which is digested to this stage in a given time by 100 cc. of raw milk.

Results.

The quantity of starch decomposed by nine samples of milk in half-an-hour and by four samples in 24 hours¹ is given in Table II.

In the control experiment with pasteurised milk it was found that 10 cc. of the milk was not able to hydrolyse more than 0.002 g. of starch (0.001 g. of starch in 10 cc. of milk cannot be detected by this method).

Table II.

(1) The quantity of starch decomposed by 100 cc. raw milk in 30 minutes at 40°.

	P.S. I	P.S. II	P.S. III	P.S. IV	P.S. V	R.S. I	R.S. II	R.S. III
	g.	g.	g.	g.	g.	g.	g.	g.
Average of 9 experiments	0.0092	0.0093	0.0096	0.0151	0.0154	0.009	0.010	0.0081
Maximum	0.011	0.011	0.011	0.021	0.021	0.010	0.012	0.010
Minimum	0.005	0.005	0.005	0.012	0.012	0.005	0.005	0.004

(2) The quantity of starch decomposed by 100 cc. of raw milk in 24 hours at 40°.

	P.S. I	P.S. II	P.S. III	P.S. IV	P.S. V	R.S. I	R.S. II	R.S. III
	g.	g.	g.	g.	g.	g.	g.	g.
Average of 4 experiments	0.0233	0.0235	0.0245	0.034	0.035	0.0233	0.0243	0.023
Maximum	0.026	0.027	0.027	0.035	0.036	0.025	0.026	0.025
Minimum	0.022	0.022	0.022	0.034	0.034	0.022	0.023	0.022

From Table II it will be seen that the eight preparations of starch employed can be arranged in the following order, according to the readiness with which they were digested:

Digestion for 30 minutes.

P.S. V; P.S. IV; R.S. II; P.S. III; P.S. II; P.S. I; R.S. I; R.S. III.

Digestion for 24 hours.

P.S. V; P.S. IV; P.S. III; R.S. II; P.S. II; (P.S. I; R.S. I); R.S. III.

In all cases, preparation P.S. V was decomposed most readily, and the digestion of R.S. III was the slowest. Preparation R.S. II which was washed with alkali was decomposed somewhat faster than P.S. III which was treated

¹ 0.3 cc. of toluene was added in these cases per 10 cc. of milk as antiseptic.

in the same way, in the course of digestion during 30 minutes. But, on the contrary, the digestion of the former preparation was rather less than that of the latter in the course of 24 hours.

We undertook the next experiment, choosing three preparations. In order to make these relationships clearer the digestion of three of the preparations P.S. III, P.S. V and R.S. II was carried out for 30 minutes, 1, 3, 5, 7, and 24 hours respectively; the results are given in Table III.

0.3 cc. of toluene was used in each experiment per 10 cc. of milk.

Table III.

The quantity of starch decomposed by 100 cc. of raw milk.

	<i>In 30 minutes.</i>			Acidity of milk N/10 alkali per 100 cc.
	P.S. III g.	P.S. V g.	R.S. II g.	cc.
Average of 8 experiments	0.0074	0.0164	0.0083	23.53
Maximum ...	0.010	0.018	0.010	23.92
Minimum ...	0.005	0.015	0.007	22.88
<i>In 1 hour.</i>				
Average of 8 experiments	0.0094	0.0244	0.0099	
Maximum ...	0.012	0.027	0.012	
Minimum ...	0.007	0.022	0.008	
<i>In 3 hours.</i>				
Average of 8 experiments	0.0123	0.0296	0.0126	
Maximum ...	0.016	0.031	0.016	
Minimum ...	0.009	0.028	0.010	
<i>In 5 hours.</i>				
Average of 8 experiments	0.0144	0.033	0.0145	
Maximum ...	0.019	0.035	0.019	
Minimum ...	0.010	0.031	0.010	
<i>In 7 hours.</i>				
Average of 8 experiments	0.015	0.0327	0.0151	
Maximum ...	0.020	0.034	0.020	
Minimum ...	0.010	0.032	0.020	
<i>In 24 hours.</i>				
Average of 8 experiments	0.0182	0.0363	0.0165	
Maximum ...	0.025	0.037	0.023	
Minimum ...	0.011	0.035	0.010	

As shown in Table III, the amount decomposed during 30 minutes was the largest in the case of P.S. V, the order being P.S. V—R.S. II—P.S. III.

This order was also maintained for the digestions of one, three, five and seven hours respectively, but was disturbed in the digestion of 24 hours by P.S. III which now came ahead of R.S. II, the order being accordingly changed to P.S. V—P.S. III—R.S. II. P.S. V was always decomposed most readily. Thus we could ascertain the fact that, at the beginning of digestion, rice-starch is decomposed a little faster than potato-starch, though the difference cannot be said to be very remarkable.

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Composition of the cheese.

The composition¹ of the samples of cheese used in this experiment was as follows (Table IV):

Table IV.

				Cheese sample I	Cheese sample II
				%	%
Water	...	...	...	34.720	36.883
Fat	...	...	...	29.623	31.175
Protein	...	...	...	31.175	27.175
Lactic acid, etc.	...	...	...	1.450	1.262
Ash	...	...	...	3.032	3.504

Preparation of a water-extract of fat-free cheese.

The following method was used for the preparation of a water-extract of fat-free cheese.

The crusts of the cheese were removed with a knife and it was then well ground in a mortar and dehydrated with alcohol. The ground cheese was extracted with ether in a Soxhlet apparatus for about three days or longer, in order to remove the fat completely. The fat-free material was then spread on a porous plate, dried at the room temperature and ground to a fine powder. 10 g. of this material were transferred to a stoppered flask containing distilled water, kept cold (7°–8°), and allowed to stand in the ice-box with frequent shaking for 1½ or 24 hours. In the latter case 1 cc. of toluene was used per 50 cc. of water as antiseptic. The solution was then filtered by suction through a Buchner funnel, containing a thin mat of long-fibred asbestos, and made up to 500 cc. with distilled water.

Method of experiment.

The experiment was carried out in the same way as that with milk, 0.3 cc. of toluene being added for 10 cc. water-extract of fat-free cheese.

As the first step; the activities of the amylase of the 24 hours' water-extract with toluene and 1½ hours' water-extract without toluene were determined, using potato soluble starch as substrate.

The results are given in Table V.

Table V.

			1½ hours' water-extract (without toluene)		24 hours' water-extract (with toluene)	
			Exp. I	Exp. II	Exp. I	Exp. II
			g.	g.	g.	g.
Time						
30 minutes	...	...	0.008	0.0095	0.003	0.006
1 hour	...	...	0.010	0.012	0.004	0.008

¹ The analyses were made by Richmond's method.

It will be seen from Table V that when the 24 hours' extract with toluene is compared with the 1½ hours' extract without antiseptics, the latter is found to be much more active than the former. Further experiments on this point were therefore considered unnecessary, and in studying amylase in cheese the 1½ hours' extract without addition of toluene was used throughout the investigation.

Results of experiments.

We investigated the digestion of the four kinds of starch P.S. III, P.S. V, R.S. II, R.S. III, comparing the quantity decomposed by cheese samples I and II during the lapse of 30 minutes, 1, 3, 5 and 24 hours respectively, and obtained the results shown in Tables VI, VII and VIII.

Table VI.

Cheese Sample I.

Grams of starch decomposed by 100 cc. of the water-extract of 2 g. fat-free cheese (=5.835 g. of original cheese at 40°).

	<i>In 30 minutes.</i>				Acidity of water-extract of fat-free cheese N/10 alkali per 100 cc.
	P.S. III g.	P.S. V g.	R.S. II g.	R.S. III g.	
Average of 19 experiments	0.0062	0.0073	0.0079	0.0054	6.60
Maximum ...	0.009	0.009	0.009	0.007	
Minimum ...	0.005	0.006	0.006	0.004	
<i>In 1 hour.</i>					
Average of 11 experiments	0.0081	0.0096	0.0108	0.0077	
Maximum ...	0.009	0.011	0.011	0.009	
Minimum ...	0.007	0.008	0.009	0.007	
<i>In 3 hours.</i>					
Average of 12 experiments	0.0141	0.019	0.0158	0.0146	
Maximum ...	0.015	0.021	0.018	0.016	
Minimum ...	0.013	0.018	0.014	0.013	
<i>In 5 hours.</i>					
Average of 5 experiments	0.0206	0.025	0.0202	0.0174	
Maximum ...	0.022	0.026	0.021	0.018	
Minimum ...	0.019	0.024	0.019	0.017	
<i>In 24 hours.</i>					
Average of 3 experiments	0.0303	0.036	0.0293	0.022	
Maximum ...	0.031	0.037	0.030	0.024	
Minimum ...	0.030	0.035	0.029	0.021	

Table VII.

Cheese Sample II.

Grams of starch decomposed by 100 cc. water-extract of 2 g. fat-free cheese (=6.519 g. of original cheese at 40°).

<i>In 30 minutes.</i>				
	P.S. III g.	P.S. V g.	R.S. II g.	R.S. III g.
Average of 7 experiments	0.0109	0.0126	0.0119	0.009
Maximum	0.012	0.013	0.013	0.010
Minimum	0.010	0.012	0.011	0.008
<i>In 1 hour.</i>				
Average of 7 experiments	0.0156	0.0174	0.0146	0.0124
Maximum	0.017	0.018	0.015	0.014
Minimum	0.015	0.016	0.014	0.012
<i>In 3 hours.</i>				
Average of 7 experiments	0.021	0.026	0.020	0.0189
Maximum	0.022	0.027	0.021	0.020
Minimum	0.020	0.025	0.019	0.018
<i>In 5 hours.</i>				
Average of 7 experiments	0.024	0.031	0.022	0.0209
Maximum	0.025	0.032	0.024	0.022
Minimum	0.023	0.030	0.022	0.019

Calculating the amount of starch decomposed by 100 g. of the original cheese from the averages given in Tables VI and VII, the following results were obtained (Table VIII):

Table VIII.

Cheese Sample I.

Time of digestion	P.S. III g.	P.S. V g.	R.S. II g.	R.S. III g.
30 mins.	0.1063	0.1251	0.1357	0.0925
1 hr.	0.1388	0.1645	0.1851	0.1320
3 hrs.	0.2416	0.3256	0.2691	0.2485
5 „	0.3530	0.4284	0.3464	0.2982
24 „	0.5193	0.6170	0.5021	0.3770

Cheese Sample II.

30 mins.	0.1672	0.1902	0.1825	0.1381
1 hr.	0.2393	0.2669	0.2240	0.1902
3 hrs.	0.3068	0.3988	0.3068	0.2899
5 „	0.3682	0.4755	0.3528	0.3206

It will be observed in Tables VI, VII and VIII that the different samples of starch decomposed by the amylase of cheese can be arranged in the following order according to the amount decomposed.

Cheese Sample I.

30 mins.	...	...	...	R.S. II	P.S. V	P.S. III	R.S. III
1 hr.	...	...	...	R.S. II	P.S. V	P.S. III	R.S. III
3 hrs.	...	...	...	P.S. V	R.S. II	R.S. III	P.S. III
5 "	...	...	...	P.S. V	P.S. III	R.S. II	R.S. III
24 "	...	...	...	P.S. V	P.S. III	R.S. II	R.S. III

Cheese Sample II.

30 mins.	...	...	...	P.S. V	R.S. II	P.S. III	R.S. III
1 hr.	...	...	...	P.S. V	P.S. III	R.S. II	R.S. III
3 hrs.	...	...	...	P.S. V	R.S. II (P.S. III)		R.S. III
5 "	...	...	...	P.S. V	P.S. III	R.S. II	R.S. III

Thus we could confirm the same facts as were found with milk that the preparation P.S. V was decomposed most easily, that the digestion of R.S. III was the worst and that the digestion of R.S. II was better than that of P.S. III.

SUMMARY.

1. The results of Spontagh, Zeitscheck, Wohlgemuth and Strich, Koning, Giffhorn, and Lane-Claypon regarding the presence of amylase in raw milk are confirmed.

2. For the quantity of soluble potato-starch decomposed by 100 cc. of raw milk, we find practically the same result as Koning and Giffhorn at half-an-hour, but more than was observed by Lane-Claypon in three hours.

3. As a result of our investigation, it appears that amylase is universally present in commercial Cheddar cheese, although the amount of this ferment varies within considerable limits.

4. Soluble potato-starch is digested more easily than potato- and rice-starch, both by milk and by the amylase of cheese.

5. Rice-starch seems to be more decomposed than potato-starch within a certain number of hours but the difference is so small as not to be distinct.

6. Soluble rice-starch is less decomposed than other starches in each experiment but it is uncertain what is responsible for this and further investigation is required.

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