BULLETIN No. 203. DECEMBER, 1901.

New York Agricultural Experiment Station.

GENEVA, N. Y.

A STUDY OF ENZYMES IN CHEESE.

L. L. VAN SLYKE, H. A. HARDING AND E. B. HART.

PUBLISHED BY THE STATION.

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SUMMARY.

I. Introduction. Cheddar cheese contains enzymes coming from (1) bacteria, (2) milk glands of cows and (3) rennet. These enzymes, or chemical ferments, change insoluble cheese-casein into soluble nitrogen-compounds. The investigation has aimed to exclude bacterial action in cheese and limit the action to results produced by enzymes present in milk when made into cheese.

II. Historical Outline. Early work done to show whether enzymes were active in cheese ripening gave negative results, owing to faulty methods of investigation. Babcock and Russell furnished the first positive evidence in 1897 in the discovery of the enzyme galactase in milk. They and others have also shown the power of rennet-enzymes to render cheese-casein soluble.

III. Methods of Chemical Analysis Used. Outlines are given to show methods used in determining total nitrogen, water-soluble nitrogen and nitrogen in forms such as albumoses, peptones, amides and ammonia; also method of determining chloroform in cheese. In milk, the term soluble nitrogen includes all nitrogen-compounds except casein and albumin; in cheese, it includes all the nitrogen soluble in water under the conditions indicated.

IV. Effect of Chloroform, Ether and Formalin on the Action of Enzymes. (1) The effect of quantities of

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Mr. L. A. Rogers, Assistant Bacteriologist, has done much of the routine work connected with the bacteriological examinations. Mr. J. A. LeClerc and Mr. A. J. Patten have rendered efficient assistance in some of the chemical work.

II. HISTORICAL OUTLINE.

The following outline of the work previously done in relation to cheese ripening covers only those features that relate to the special problems we have been studying.

In 1887, Benecke,[1] in discussing the rôle of bacteria in cheese ripening, stated that, while they probably caused such changes, yet the ripening might really be due to the activity of some unorganized ferment. He pointed out that, if bacteria are not essential to cheese ripening, this fact could be made clear by the preparation of cheese under conditions which would exclude bacterial activity. Acting on this suggestion Adametz[2] prepared a number of *Hauskäse*, a form of soft cheese, in the normal way, except that he added various disinfectants to the milk or to the curd derived from it.

When cheeses made with the addition of kreolin or of thymole, were examined bacteriologically, they were pronounced sterile; but even when kept for double the normal length of time, they did not take on the appearance of ripened cheese. His experiments with salicylic acid, oxalic acid and with vapors of carbon disulphide and iodine were less satisfactory in repressing the microörganisms in the cheese, but these agents seemed to hold back the ripening in proportion as they inhibited the activity of germ life.

Cheese investigators quite generally accepted these results as settling the point raised by Benecke, and during the succeeding ten years the work on cheese ripening was based upon the theory of germ action.

The phenomena of ripening in cheese may be divided into two classes, (1) the chemical decomposition of casein and (2) the formation of cheese flavors. These may or may not arise from

[1] Benecke. Cent. f. Bak., I Abt., **1**: 521 (1887).

[2] Adametz. Thiel's Landwirtschaftliche Jahrb., **18**: 227 (1889).

common causes. The casein begins to undergo change at once, while the formation of flavors begins some time later, after which the two progress simultaneously. These two groups of phenomena cannot be measured by the same means or standards.

Duclaux, Adametz, Weigmann and their disciples have directed their attention to the formation of flavors and have quite generally relied upon the odor and physical appearance of their material in judging of the rate and character of the ripening. In those cases in which they have gone more fully into the solubility of the casein, they have usually determined this point by its ability to pass through a porcelain filter, a method which von Freudenreich & Jensen[3] have shown to be extremely liable to error in practice. They have rarely attempted to show that the species of bacteria which they look upon as the causal ones are present in cheese in any considerable quantities. They have, for the most part, confined themselves to showing that pure cultures of these species are able, by means of excreted enzymes, to digest the casein of milk and at the same time to form cheese-like odors. In some cases they have made cheese with the addition of pure cultures or of solutions of their enzymes and have stated that the resulting product was better flavored than cheese made in the usual way. In establishing this point, however, they were handicapped by the lack of accurate standards for measuring such relations.

Recently Adametz and Winkler[4] have placed a culture of one of these bacilli upon the market under the trade name of "Tyrogène," its use being expected to result in the production of a desirable Emmenthaler flavor in cheese. Some preliminary tests by von Freudenreich[5] have failed to indicate that it will accomplish this desired end.

When the study of the kinds of bacteria present in cheese was extended so as to include the numbers of each kind, it was found that the enzyme-forming bacteria previously mentioned were present only in small numbers. Even when large numbers were added to milk before making it into cheese, these bacteria ceased to

[3] v. Freudenreich and Jensen. Landw. Jahrb. d. Schweiz., **14**: 169 (1899).
[4] Winkler. Molkerei Ztg., **14**: 817 (1900).
[5] v. Freudenreich. Ann. Agr. Suisse (1901).

grow almost as soon as the curd was put into the press and rapidly disappeared in the cheese.

It was found that from the time cheese was made until fully ripened there were present few beside *lactic acid* bacteria, so called because they curdle milk by production of acid without subsequent digestion of the casein. From these results von Freudenreich was led to question the connection between enzyme-forming bacteria and the ripening process. He made numerous attempts to produce cheese with the addition of cultures of enzyme-forming bacteria, which uniformly resulted in a product of poorer flavor, according to his opinion. He then became the champion of the theory that lactic acid bacteria are the principal, if not the only, cause of cheese ripening.[6]

The chief objection to this theory is the fact that no one has yet been able to demonstrate the production of an enzyme on the part of lactic acid bacteria. Without such aid it is difficult to understand how a bacterium is to attack an insoluble substance such as the coagulated casein in cheese.

Von Freudenreich[7] added chalk to milk cultures of these lactic acid bacteria for the purpose of preventing the accumulation of acid and of simulating in this respect the conditions found in cheese. He was thus able to demonstrate the ability of these organisms to increase materially the amount of soluble nitrogen. However, Chodat and Hoffman-Bang[8] have pointed out that this is not equivalent to attacking the casein after it has been coagulated by rennet. They maintain that lactic acid bacteria are unable to attack coagulated casein, even when sugar is not present.

In a later publication Jensen,[9] without bringing forward adequate experimental evidence, has suggested that lactic acid bacteria are able to elaborate an enzyme.

In 1897 Babcock & Russell[10] announced that the milk of all mammals contains, in addition to the previously known substances, *galactase*, a tryptic-like ferment capable of producing

[6] v. Freudenreich. Cent. f. Bak., II Abt., **1**: 384 (1895).
[7] v. Freudenreich. Cent. f. Bak., II Abt., **3**: 231 (1897).
[8] Chodat and Hoffman-Bang. Ann. Inst. Pasteur, **15**: 36 (1901).
[9] Jensen. Landw. Jahrb. d. Schweiz., **14**: 197 (1900).
[10] Babcock and Russell Ann. Rept. Wis. Exp. Sta. **14**: 161 (1897).

digestion of casein, and they suggested that this substance might play a considerable role in cheese ripening. The correctness of their statements regarding the existence of this substance has been substantiated by Storch,[11] von Freudenreich[12] and Jensen.[13]

In 1900 Babcock, Russell and Vivian[14] and Jensen[15] almost simultaneously called attention to the ability of pepsin, contained in rennet solution, to render casein soluble, and they presented experimental evidence to establish this point.

Thus, we see that cheese, as ordinarily manufactured, contains enzymes derived from three different sources, (1) bacteria, (2) milk glands of cows and (3) rennet.

Enzymes, in acting upon casein, cause its decomposition and probably produce compounds that furnish some of the cheese flavors. While we appreciate as highly important, from a practical standpoint, the study of cheese flavors, we have devoted our time chiefly to a study of enzyme action upon cheese-casein. It seems that this constitutes so fundamental a problem in cheese ripening that it should be first studied, and moreover its solution will doubtless go far toward solving the problem of flavors.

III. METHODS OF CHEMICAL ANALYSIS USED.

In a later bulletin the methods of chemical analysis used in determining the amounts of nitrogen present in different forms in cheese will be presented and discussed in full detail. In this connection it seems sufficient to present only a brief outline of such methods.

PREPARATION OF CHEESE EXTRACT.

Twenty-five grams of cheese are mixed with quartz sand and treated at 122° to 140° F. (50 to 60° C.) for a half hour with each of several successive portions of water, decanting and filter-

[11] Storch. 40 Rept. Copenhagen Exp. Sta (Denmark).

[12] v. Freudenreich. Cent. f. Bak., II Abt., **5**: 241 (1899).

[13] Jensen. See Footnote 9.

[14] Babcock, Russell and Vivian Ann. Rept. Wis. Exp. Sta., **17**: 102 (1900), also Cent. f. Bak., II Abt , **6**: 817 (1900).

[15] Jensen. Landw. Jahrb. d. Schweiz., **14**: 197 (1900), also Cent. f. Bak., II Abt., **6**: 734 (1900).

ing each portion of extract until 500 cc. have been accumulated. Portions of the solution thus prepared are used in making the various determinations.

DETERMINATION OF NITROGEN-COMPOUNDS IN CHEESE EXTRACT.

(a) *Total water-soluble nitrogen* is determined in an aliquot part of the water extract.

(b) *Precipitation by alum.*—To 100 cc. of water extract, 2 cc. of saturated alum solution are added and digested at 104° to 108° F. (40° to 42° C.) until precipitation is complete. The precipitate is filtered, washed and then treated by Kjeldahl method to determine nitrogen.

(*c*) *Coagulation by neutralizing and boiling.*—The clear filtrate from (b) is exactly neutralized by dilute fixed alkali and heated on water bath until coagulation is complete. The precipitate is filtered, washed and its nitrogen determined by Kjeldahl method.

(d) *Albumoses.*—To the filtrate from (c) two or three drops of dilute (one to three) sulphuric acid are added, and then powdered zinc sulphate to saturation. The mixture is heated on water bath until precipitation is complete and the nitrogen is determined in the precipitate washed with saturated solution of zinc sulphate.

(e) *Peptones.*—To the filtrate of (d) two or three drops of strong hydrochloric acid are added and then bromine in successive portions of a few drops at a time, accompanied by vigorous shaking until the liquid becomes super-saturated. The nitrogen in the washed precipitate is determined as before.

(f) *Amides.*—(1) First Method. The nitrogen in filtrate from (e) is determined directly by Kjeldahl method and this, less the nitrogen present as ammonia, is the amide nitrogen.

(2) Second Method. To 100 cc. of the original cheese extract there is added about one gram of common salt, together with an excess of ten per ct. tannic acid solution. The precipitate formed is filtered and washed and the nitrogen determined in an aliquot part of the filtrate. From this amount of nitrogen is deducted the amount of nitrogen found as ammonia in (g) and the remainder is the amount of amide nitrogen.

(g) *Ammonia.*—To an aliquot portion of the filtrate obtained in (f), magnesium oxide is added and the ammonia separated by distillation.

DETERMINATION OF NITROGEN-COMPOUNDS IN MILK.

Casein.—To 20 gms. of milk, diluted with water to about 100 cc., are added 2 to 2½ cc. of saturated alum solution. The determination is completed as under (b) in cheese extract, and the other determinations are made as described above in the cheese extract.

DETERMINATION OF CHLOROFORM IN CHEESE AND MILK.

About 5 gms. of milk or cheese are placed in a pressure bottle with about 10 cc. of alcohol and 5 gms. caustic potash. The bottle and contents are then heated 30 minutes at 230°F. (110°C.) in an autoclave. The resulting chloride is determined volumetrically as in case of chlorine in sodium chloride.

FORM OF STATING RESULTS.

The figures given in the various tables represent percentages of the total nitrogen in milk and cheese. This form of statement is usually preferable, as figures representing the actual percentages in milk and cheese are often very small. Hence, considerable variations, expressed in percentages of nitrogen, often represent very small variations when expressed in actual amounts present in cheese and milk.

The soluble nitrogen in milk, as used in this bulletin, includes all nitrogen compounds except casein and albumin. The water-soluble nitrogen in cheese includes all the nitrogen soluble under the conditions indicated in preparing the water extract of cheese.

IV. EFFECT OF CHLOROFORM, ETHER AND FORMALIN ON THE ACTION OF ENZYMES.

In an investigation of this kind a prime necessity is a means of totally suppressing the action of germ life. It is equally important that the action of the agents employed shall not be so violent as to alter the enzymes or the casein.

The work of Babcock and Russell has suggested two substances suitable for this purpose, ether and chloroform. Of the two we have used chloroform almost exclusively for several reasons:

(1) As an anæsthetic it is more efficient; (2) its proportion in any mixture can be quantitatively determined with approximate accuracy by chemical analysis; (3) the amount required to prevent germ growth does not so largely increase the bulk of the mixture; (4) being less volatile, there is less loss in sampling materials under investigation; (5) it is not inflammable.

In all our work with solutions it has been our aim to mix carefully by shaking at least once a day during the entire course of the experiment. Too much stress cannot be laid upon this point since mixtures of milk with ether or chloroform tend to separate on standing and thereby produce conditions favoring the germination of spores in certain portions of the mixture.

EFFECT OF VARYING PERCENTAGES OF CHLOROFORM ON ENZYME ACTIVITY.

Since the relation of chloroform to the activity of these enzymes has not been investigated, except in a very general way by Babcock and Russell, the following study of its action on galactase and bacterial enzymes was made.

Duplicate bottles of separator skim-milk containing only a trace of fat were prepared containing 2.5, 5, 10, 20 and 30 per ct. of chloroform by volume. These bottles were kept at 60° F. (15.5° C.), and examined both chemically and bacteriologically.

TABLE I.—INFLUENCE OF VARYING AMOUNTS OF CHLOROFORM UPON THE ACTIVITY OF ENZYMES.

Amount of chloroform.	Age.	In 100 lbs. total nitrogen.			No. of germs per
		Total soluble nitrogen.	Nitrogen in albumoses and peptones.	Nitrogen in amides.	
Per ct.	*Days.*	*Lbs.*	*Lbs.*	*Lbs.*	
	fresh	9.33	4.58	4.75	———
2.5	7	11.60	6.81	4.79	28
5	7	11.63	6.94	4.69	46
10	7	11.72	7.07	4.65	26
20	7	11.63	6.79	4 84	25
30	7	12.28	7.37	4.91	18
2.5	21	16.80	9.94	6.86	42
5	21	16.16	8.47	7.69	36
10	21	16.39	8.99	7.40	25
20	21	16.37	8.34	8.03	26
30	21	13.69	7.07	6.62	30
2.5	49	21.79	14.75	7.03	———
5	49	21 65	14.63	7.02	———
10	49	21.40	14.93	6.47	———
20	49	20.11	14.36	5.75	———
30	49	22.99	16.17	6.82	———
2.5	112	33.15	18.81	14.34	9
5	112	33.62	18 59	15.03	10
10	112	30.51	16.38	14.13	11
20	112	33.78	16.39	17.39	6
30	112	33.06	19.15	13.91	5
2.5	192	41.98	19.18	22.80	13
5	192	39.37	14.85	24 52	14
10	192	35.36	15.13	20.23	7
20	192	35.65	16 37	19.28	6
30	192	35 78	17.30	18.48	———

During 112 days the amount of soluble nitrogen varied within very narrow limits in the different bottles. During the next 80 days the bottles containing 2.5 and 5 per ct. of chloroform showed a little more soluble nitrogen than the others, in which the amounts of soluble nitrogen were almost identical. These results could hardly be interpreted as indicating, even after the lapse of 192 days, any marked difference in the effect of definite quantities of chloroform upon the activity of enzymes. The germ content, as shown by bacteriological analysis, is in entire agreement with the results of chemical analysis.

From these results we see that in the presence of 2.5 per ct. of chloroform the increase of soluble nitrogen is continuous and considerable. However, these results do not enable us to know whether the chloroform exercised any restraining influence upon the activity of the enzyme. Any such repressing effect of chloroform upon enzyme action could be directly shown only by using as a means of comparison milk containing no chloroform but under such conditions the action of bacteria would render the comparison worthless.

A comparison of the changes produced in the bottles containing the different percentages of chloroform shows a surprisingly small decrease of change in bottles having the larger proportions of chloroform. This tends to show that chloroform restrains enzyme action only slightly.

The germ content, even in the bottles containing only 2.5 per ct. of chloroform, was so small that the observed changes were undoubtedly due to the enzymes present in the milk at the beginning of the experiment.

EFFECT OF VARYING PERCENTAGES OF FAT UPON THE ANTISEPTIC VALUE OF CHLOROFORM.

In the case of ether, Babcock and Russell[16] have shown that it has a strong tendency to combine with the fat present in such a way as not to exert its anæsthetic influence. For this reason rich cream could hardly be kept from decomposing through bacterial action when ether was used.

To test this phase of the question with chloroform, two series of bottles were prepared. The first contained 10 per ct. and the second 20 per ct. of butter-fat and in each series duplicate bottles contained 2.5, 5, 10 and 20 per ct. of chloroform.

In order that the transformations in each of the bottles in the two series should be directly comparable when expressed in percentages of total nitrogen, it was necessary that for a given quantity of nitrogen in any bottle there should also be present a corresponding amount of enzyme.

In order to maintain these relations, each bottle contained 900 cc. of a mixture made up of 540 cc. of whole milk, together with

[16] Babcock and Russell. See Footnote 10.

sufficient chloroform and melted butter-fat to give the desired percentages by volume. Water was then added to bring the total up to 900 cc.

The butter-fat used, after being heated above 185° F. (85° C.) for 10 minutes to kill the enzymes present, was filtered to remove the coagulated casein and was then decanted to free from water and salt.

The chloroform assisted in emulsifying the fat and it was only in those bottles containing the smaller percentages of chloroform that difficulty was experienced in getting satisfactory samples for chemical analysis. In order to minimize this difficulty, the bottles were warmed at 99° F. (37° C.) for a few hours before sampling in order to melt the fat. During the rest of the time they were kept at 60° F. (15.5° C.).

TABLE II.—EFFECT OF VARYING AMOUNTS OF FAT UPON THE ANTISEPTIC VALUE OF CHLOROFORM.

Proportions of		Age.	In 100 lbs. total nitrogen.			Number of germs per cc.
Chloroform.	Fat.		Total soluble nitrogen.	Nitrogen in albumoses and peptones.	Nitrogen in amides.	
Per ct.	*Per ct.*	*Days.*	*Lbs.*	*Lbs.*	*Lbs.*	
		Fresh:				17,124
20	10	14	19.31	10.52	8.79	———
20	20	14	22.35	10.07	12.28	———
10	10	14	21.92	12.86	9.06	———
10	20	14	19.47	9.38	10.09	———
5	10	14	24.45	15.09	9.36	———
5	20	14	23.58	12.23	11.35	———
2.5	10	14	28.14	16.21	11.93	———
2.5	20	14	28.82	15.65	13.17	———
20	10	56	35.47	21.56	13.91	165
20	20	56	34.85	20.04	14.78	163
10	10	56	38.06	23.20	14.86	167
10	20	56	38.23	21.26	16.97	———
5	10	56	39.24	23.03	16.21	113
5	20	56	39.48	22.36	17.12	82
2.5	10	56	38.26	24.44	13.82	209
20	10	112	34.66	18.28	16.38	298
20	20	112	36.74	20.24	16.50	———
10	10	112	37.08	18.62	18 46	260
10	20	112	41.78	21.40	20.38	142
5	10	112	40.82	19.35	21.48	196
5	20	112	43.05	23.95	19.10	107
2.5	10	112	36.48	18.53	17.95	274
2.5	20	112	38.81	19.09	19.72	34

The preceding table does not show any marked influence due to the presence of such varying amounts of fat.

There are more bacterial spores present than are shown in the results given in Table I. This is probably due to the combined action of a number of factors: (1) The heating of the butter was not high enough to kill the spores introduced from that source; (2) the presence of many small globules of fat in the cultures makes counting difficult and tends to give too high figures; (3) this experiment was started in midsummer, when the air is better supplied with spores than in midwinter when the former investigation was begun.

A comparison of the percentages of change shown in Tables I and II after corresponding intervals shows the transformation to have been more rapid in the case of Table II. This is easily accounted for by the fact that here whole milk was used and the proportion of enzyme to nitrogen was greater than in the former case where the skim-milk was poorer in enzyme on account of the amount lost in the separator slime and in the cream.

COMPARISON OF EFFECT OF ETHER, CHLOROFORM AND A MIXTURE OF BOTH UPON ENZYME ACTION.

Milk was obtained from two cows, care being taken to brush and moisten the flank and udder and to steam the pail, but by mistake the fore-milk was used in the case of one cow. The milk was taken directly to the laboratory and plates made, which later showed a germ content of 2719 per cc. The fat content of the milk was 4.5 to 5 per ct. Duplicate bottles were prepared in three series containing (1) 15 per ct. of ether, (2) 3 per ct. chloroform and (3) a mixture containing 2.9 per ct. of ether and 2.1 per ct. chloroform. The bottles were kept at 99° F. (37° C.).

TABLE III.—COMPARISON OF EFFECTS OF ETHER, CHLOROFORM, AND MIXTURE OF BOTH UPON THE ACTIVITY OF ENZYMES.

Anæsthetic used.				Age.	In 100 pounds total nitrogen.				Number of germs per cc.
		Mixture.			Total soluble nitrogen.	Nitrogen in albumoses.	Nitrogen in peptones.	Nitrogen in amides.	
Ether.	Chloroform.	Ether.	Chloroform.						
Per ct.	*Per ct.*	*Volumes.*		*Days.*	*Lbs.*	*Lbs.*	*Lbs.*	*Lbs.*	
				Fresh					2719
15	.....	.....		2	11.92	3 95	2.93	5.04	132
	.....	3	2	2	13.61	4.54	3.87	5.19	140
	3	.....		2	13.18	5.66	2.38	5,14	93
15	.....	.....		5	16.75	4.81	5.64	6.30	19
	.....	3	2	5	14.90	4.26	4.25	6.39	116
	3	.....		5	13.23	3.70	3.60	5.93	28
15	.....	.....		8	20.73	6.38	5.65	8.70	——
	.....	3	2	8	17.67	5.18	4.71	7.78	——
	3	.....		8	17.31	4.26	4.62	8.43	——
15	.....	.....		14	25.64	9.90	4.72	11.02	100
	.....	3	2	14	25.92	8.98	3.98	12.96	5
	3	.....		14	24.82	9.16	3.61	12.04	6
15	.....	.....		21	32.49	14.34	4.90	13.25	121
	.....	3	2	21	29.71	11.29	4.53	13.89	62
	3	.....		21	27.89	10.55	4.81	12.50	6
15	.....	.....		28	35.81	14.89	8.42	12.50	161
	.....	3	2	28	32.12	10.74	6.85	14.55	18
	3	.....		28	29.80	11.66	5.64	12.50	4
15	...	.....		35	36.47	15.18	7.31	13.89	——
	.....	3	2	35	32.49	13.24	5.46	13.79	11
	3	.....		35	30.36	12.22	6.01	12.13	5
15	.....	.....		42	39.63	18.98	7.87	12.78	138
	.....	3	2	42	36.57	15.56	7.59	13.42	13
	3	.....		42	35.36	15.74	7.61	12.00	6
15	.....	.....		56	45.75	19.45	11.39	14.91	183
	.....	3	2	56	40.28	17.50	10.83	11.95	5
	3	.....		56	Lost.				
15	.....	.....		84	55.01	22.22	15.38	17.41	320
	.....	3	2	84	48.44	17.88	12.60	17.96	4
	3	.....		84	45.83	18.52	10.83	16.58	3
15	.....	.....		137	60.74	15.43	24.03	21.28	113
	.....	3	2	137	57.33	14.73	24.08	18.52	2
	3	.....		137	Lost.				1

From these results it is seen that a slightly greater amount of soluble nitrogen was formed in the presence of 15 per ct. of ether than under either of the other two conditions. From this it might be inferred that 15 per ct. of ether was more favorable to enzyme action that 3 per ct. chloroform, but the results of the bacteriological analyses give some reason for believing that there had taken place a growth of bacteria. There had probably been a corresponding increase in the amount of bacterial enzyme. This is rendered more likely by the fact that the bacteria in this case were almost entirely of a single kind, which showed ability to grow in the presence of ether, formed spores quickly in almost every cell and elaborated enzyme with great freedom.

This experience has made us slow to accept as trustworthy any results obtained with the use of ether, when the conditions are not constantly controlled by quantitative examination of the bacterial content.

COMPARISON OF EFFECTS OF CHLOROFORM AND FORMALIN UPON ACTIVITY OF ENZYMES.

Jensen[17] in a suggestive article on the enzymes of cheese ripening has called attention to the use of 0.1 per ct. of formalin in studying their activity. Babcock and Russell[18] have stated that comparatively small amounts of this substance completely inhibit enzyme activity. The use of even the amounts recommended by Jensen is to be looked upon with suspicion until the influence of formalin upon enzyme action is more fully investigated.

In order to facilitate comparisons at some future time, we give the results of parallel examinations of four samples of milk containing respectively 4 per ct. of chloroform and 0.1 per ct. of formalin by volume. Unfortunately the strength of formalin was not redetermined but it was the 40 per ct. article of commerce.

The milk in this case was obtained from the four quarters of a single cow at one milking. The flank and udder were brushed and moistened. The hands of the milker were smeared with vaselin and the milk was caught in four-inch glass funnels leading into glass bottles, all of which had been carefully steamed. The milk was taken at once to the laboratory and placed under the influence of chloroform and formalin at 99° F. (37° C).

[17] Jensen. See No. 9.

[18] Babcock and Russell. Ann. Rept. Wis. Exp. Sta. **15**: 77 (1898).

TABLE IV.—COMPARISON OF EFFECTS OF FORMALIN AND CHLOROFORM UPON ACTIVITY OF ENZYMES.

MILK DRAWN OCT. 10.

Germicide used.		Age.	In 100 lbs. total nitrogen.			Number of germs per cc.
Formalin 0.1 per ct.	Chloroform. 4 per ct.		Total soluble nitrogen.	Nitrogen in albumoses and peptones.	Nitrogen in amides.	
		Days. Fresh	*Lbs.*	*Lbs.*	*Lbs.*	87
I	.....	14	37.50	20.47	17.03	1
	II	14	53.09	23.84	29.25	51
I	.....	42	50.26	28.19	22.07	0
	II	42	64.22	24.57	39.65	38
I	.....	77	59.18	35.87	23.31	1
	II	77	72.57	33.63	38.94	5
I	.....	152	69.94	22.12	47.82	*
	II	152	67.62	43.58	24.04	20
		Fresh	———	———	———	6
III	.....	14	21.28	12.81	8.47	0
	IV	14	35.46	21.97	13.49	9
III	.....	42	23.01	13.73	9.28	6
	IV	42	36.56	21.36	15.20	4
III	.....	77	24.32	16.67	7.65	0
	IV	77	42.96	27.78	15.18	1
III	.....	152	24.67	17.70	6.97	1
	IV	152	43.59	31.40	12.19	2
		Fresh	———	———	———	232
V	.....	14	37.42	20.98	16.44	0
	VI	14	49.83	25.63	24.20	3
V	.....	42	42.89	24.96	17.93	3
	VI	42	63.58	27.36	36.22	18
V	.....	77	53.50	31.95	21.55	2
	VI	77	68.50	31.12	37.38	1
V	.....	152	48.70	27.96	20.74	0
	VI	152	63.89	36.10	27.79	0

*Bacteria present in large numbers.

NOTE.—I and II, front right quarter; III and IV, front left; V and VI, back right; VII and VIII, back left.

TABLE IV.—*Continued.*

Germicide used.		Age.	In 100 lbs. total nitrogen.			Number of germs per cc.
Formalin 0.1 per ct.	Chloroform. 4 per ct.		Total soluble nitrogen.	Nitrogen in albumoses and peptones.	Nitrogen in amides.	
		Days. Fresh	*Lbs.*	*Lbs.*	*Lbs.*	138
VII	....	14	32.41	19.63	12.78	3
	VIII	14	50.67	29.42	21.25	3
VII	.....	42	40.12	27.47	12.65	2
	VIII	42	57.23	29.72	27.51	1
VII	.....	77	47.00	29.40	17.60	0
	VIII	77	64.38	39.88	24.50	0
VII	.....	152	49.91	34.37	15.54	0
	VIII	152	62.48	39.75	23.73	3

From the above results we see that the number of bacteria in all the bottles remained very low. In all cases the decomposition has gone on more slowly in the presence of formalin than with chloroform, as is clearly shown by the following tabulated summary of results.

TABLE IV A.—AVERAGE OF FOUR QUARTERS.

Age.	Total soluble nitrogen.	
	With formalin.	With chloroform.
Days.	*Per ct.*	*Per ct.*
14	32.15	47.26
42	39.07	55.39
77	46.00	62.10
152	45.62	60.63

In the article by Jensen previously referred to, he notes the same relation in the action of these two substances and he is inclined to hold the view that 0.1 per ct. formalin completely inhibits the action of galactase but allows bacterial enzymes to work. If this view is correct, we must consider that over 70 per ct. of the

decomposition here produced in the presence of chloroform is caused by enzymes other than galactase.

It seems hardly possible that sufficient bacterial enzyme could have been formed in the cases of No. III to account for the changes observed in the presence of formalin. The milk in this quarter of the udder was unusually free from bacteria, having been caught under most favorable conditions and placed under the influence of formalin within a few minutes.

CONNECTION BETWEEN BACTERIA IN THE UDDER AND ENZYMES IN THE MILK.

Previous investigators[19] have noted that there is considerable difference in the rate of change caused by enzymes in different samples of freshly drawn milk. These differences have been attributed to variations in the enzyme-forming activity of the milk glands but we have been led to look for another explanation of these irregularities. The production of enyzmes on the part of certain classes of bacteria is well known but the bacterial formation of enzymes in the udder, able to perform work in cheese ripening, is a possibility which has not been seriously considered.

The work of Ward[20] has called attention to the fact that in many cases the interior of the udder is inhabited by certain microörganisms which find the conditions favorable to their continued development. In working with certain Station cows we have found that in some cases large numbers of germs were present in the milk last drawn. This condition existed whenever examinations were made during a period of some months.

By comparing the germ content of the whole mess of milk, after rejecting the milk first drawn, with the germ content of the milk last drawn, or strippings, it is often found that the number present in the whole mess exceeds that in the strippings by an amount hardly larger than would be expected as a result of unavoidable contamination during milking.

This is shown in the following table which gives the number of bacteria found per cubic centimeter in the whole mess and in

[19] Babcock and Russell. See No. 10.

[20] Ward. Bul. No. 178 Cornell Exp. Sta. (1900).

the strippings from each quarter of a single cow at three successive milkings. In all cases the first few streams from each quarter were rejected.

TABLE V.—NUMBER OF BACTERIA PER CUBIC CENTIMETER IN WHOLE-MILK AND STRIPPINGS.

Date.	Front left quarter.		Front right quarter.		Back left quarter.		Back right quarter.	
	Whole-milk.	Strip-pings.	Whole-milk.	Strip-pings.	Whole-milk.	Strip-pings.	Whole-milk.	Strip-pings.
June 11, P.M.....	53	26	——	56	140	173	401	716
June 12, A.M.....	646	244	216	429	442	493	629	1870
June 12, P.M.....	88	22	36	305	96	105	789	975

These data strongly support the idea that the interior of the udder in such cases is seeded with these organisms, which are generally yellow cocci, capable of liquefying gelatin.

Most striking are those cases in which the interior of certain quarters of the udder is highly contaminated with certain organisms for long periods, while, at the same time, one or more quarters of the udder in the same animal may remain comparatively free from germ life. In the case of the cow used in collecting the data shown in the above table, examinations of the strippings were made extending over four months. Samples were collected by catching one of the last streams from each quarter in a sterile test tube, except in a few cases in which they were drawn with a sterile milking tube. The samples were taken at once to the laboratory and plates prepared containing 1 cc. and 0.5 cc. of the milk. The results are shown in the following table:

TABLE V A—NUMBER OF BACTERIA PER CUBIC CENTIMETER IN THE STRIPPINGS OF COW No. 8.

Date.	Front left quarter.	Front right quarter.	Back left quarter.	Back right quarter.
May 26	———	22	296	372
June 11, P. M.	26	56	173	716
June 12, A. M.	244	429	493	1870
June 12, P. M.	22	305	105	975
July 12	22	48	2106	488
Jnly 18	36	55	280	868
July 27	211	10	388	628
Sept. 7	391	3694	631	656
Sept. 20	132	450	356	9967
Oct. 10	6	87	138	232

This table shows that in general the strippings from the back right quarter had a germ content of 500 to 800 per cubic centimeter; the back left quarter had slightly less; the front left quarter had often less than 100 per cubic centimeter, and the front right quarter but little more.

Making allowance for the work done by galactase, the milk from different quarters of the udder of the above mentioned cow should show different rates of chemical change proportional to the number of germs present in the respective quarters of the udder, if these changes are to be associated with contamination within the udder.

The results already given in Table IV, under chloroform relate to this point. The quarters of the udder are there designated as follows: II, front right; IV, front left; VI, back right; VIII, back left. The second determination was made in the presence of 4 per ct. of chloroform. In order to obtain sufficient material for a large number of analyses, three successive messes of milk were collected and united. Care was taken to reject the fore-milk and keep out bacteria from other sources. The following table shows the results in this test up to 15 weeks:

TABLE V B. — SOLUBLE NITROGEN FORMED IN MILK FROM DIFFERENT QUARTERS OF UDDER.

MILK DRAWN JUNE 11 AND 12.

Age of milk when analyzed.	Soluble nitrogen in 100 lbs. total nitrogen.			
	Front left quarter.	Front right quarter.	Back left quarter.	Back right quarter.
Days.	*Lbs.*	*Lbs.*	*Lbs.*	*Lbs.*
7	27.01	29.23	48.23	46.66
21	36.25	39.75	56.61	56.66
35	36.65	40.60	59.37	57.54
49	lost	40.48	60.68	59.63
105	"	55.31	77.29	71.63

The results given in Tables IV and V B, show in a general way that there is a relation between the numbers of bacteria present in the udder and the rapidity with which the milk produced there undergoes self-digestion in the presence of chloroform or formalin.

It may be held that the presence of these bacteria has merely stimulated the production of an extra amount of galactase, but many of these bacteria are able to bring about the liquefaction of gelatin, a fact which suggests that they have played a part in enzyme formation within the udder. However, it is impossible to assign even an approximate value to the work performed by bacteria within the udder in the production of their enzymes, until we understand the conditions which relate to the normal formation of galactase.

V. COMPARISON OF RIPENING PROCESS IN CHEESE MADE WITH CHLOROFORM AND IN NORMAL CHEESE.

Previous attempts to study the part played by enzymes in cheese ripening have proceeded indirectly by a study of enzyme action in milk or have been carried out with cheese in a fragmentary manner. In addition to the early work of Adametz, Babcock and Russell report that they have observed the changes that have taken place at the end of about a year in a cheese containing chloroform. They also added rennet to milk containing ether and determined the general changes taking place in the

coagulum. Jensen[21] also reports the changes taking place in a cheese to which he had added trypsin and ether. However, so far as we can learn, no cheese has, hitherto, been prepared under conditions essentially normal except for the presence of an anæsthetic, and been kept for a long period completely under the influence of that anæsthetic, with systematic chemical and bacteriological examinations at frequent intervals.

METHOD OF MANUFACTURE AND SAMPLING.

The preparation of a chloroform cheese presents no extreme difficulties. Chloroform added directly to the milk tends to settle to the bottom but the stirring which accompanies the manufacture serves to keep it distributed without any considerable loss from evaporation. The addition of rennet at 84 to 88° F. (29 to 31° C.), cutting and heating to 98 to 100° F. (37 to 38° C.), proceed in the usual way, except that both the curdling of the milk and the expulsion of whey take place more slowly than in normal cheese. The expulsion of the whey is especially prolonged because of the absence of acid, and the moisture content of the resulting cheese may be somewhat higher than in a first-class normal Cheddar. After the whey is drawn and the curd is fairly well drained, it is put to press with or without previous salting.

In making more than a dozen of these cheeses at different times, we have added to the milk from 2 to 5 per ct. of chloroform by volume and we find that the percentage of chloroform by weight in the resulting cheese mass is about three times the figure given for the milk.

The cheese is kept continuously under pressure 18 to 24 hours and is then transferred to a room with a temperature varying only one or two degrees from 60° F. (15.5° C.) and placed under a bell jar in an atmosphere of chloroform. The moisture of cheese under bell jars remains fairly uniform.

After testing a number of receivers we have settled upon bell-jars, or carefully soldered cans which are inverted over the cheese, and fit into a groove in a heavy wooden base. The base is first boiled in paraffin to fill all the pores and melted paraffin is used

[21] Jensen. Tidskr. for Fysik. og Kemi, 2: 92–114 (1897).

as a seal in fastening the cover into the grooves, thus reducing the loss of chloroform and moisture to insignificant amounts.

At regular intervals the cover is removed and samples taken with a sterilized tryer for chemical and bacteriological analysis. The former includes a quantitative determination of the chloroform present in the cheese. To replace the small amounts lost by leakage and evaporation, measured amounts of chloroform are added to a dish within the container at the time of each examination.

DECOMPOSITION IN CHEESE UNDER CHLOROFORM COMPARED WITH THAT IN NORMAL CHEESE.

In order to get an idea of the changes brought about by the combined influence of all the enzymes present at the time a cheese is made, 3.5 lbs. of chloroform were added to 125 lbs. of night's and morning's milk, having the degree of acidity suitable for Cheddar cheese-making. One-half ounce of Hansen's liquid rennet was added at 88° F. (31° C.), and the cheese made as described above. One-half of the resulting curd, without salting, was pressed into form of a Young America cheese. On the third day it was found to contain 35 per ct. of water and 15 per ct. of chloroform.

As a basis for comparison there is also given the analysis of a normal cheese ripened at the same temperature and having originally about the same percentage of moisture. However, since under normal conditions the moisture in a cheese rapidly decreases, while in the chloroform cheese this factor remains practically constant, there is also given the analysis of a cheese normal in every way except that it was coated with a layer of paraffin to lessen the loss of moisture.

TABLE VI.—COMPARISON OF NORMAL CHEESES, CURED WITH AND WITHOUT PARAFFIN COVERING, WITH A CHEESE MADE AND CURED WITH CHLOROFORM.

Conditions of curing.	Total water-soluble N. formed for 100 lbs. N. in cheese.					
	2 weeks.	1 month.	2 months.	6 months.	12 months.	15 months.
Cheese No. 31 A, cured under normal conditions	11.50	18.50	25.10	33.70	37.30	38.66
Cheese No. 31 B, covered with paraffin	12.50	19.30	25.40	37.80	40.90	44.14
Cheese No. 30 A, made and cured with chloroform . .	5.30	5.70	8.20	14.50	22.60	27.70

In Tables VI, VII, VIII and IX, the figures given for total water-soluble nitrogen represent the amount rendered soluble after the cheese was taken from the press. Samples of the green cheese fresh from the press were analyzed, and it was found that the amount of soluble nitrogen varied considerably in different cheeses. Therefore, for the sake of more accurate comparison, the amounts of water-soluble nitrogen found in the green cheese have been deducted and so are not included in the figures presented in these tables.

The data in Table VI show that at the end of one month the water-soluble nitrogen in the normal cheese was more than three times that contained in the chloroform cheese; gradually the difference decreased until at the end of 15 months the total decomposition in the case of the chloroform cheese amounted to 27.7 per ct. of the total nitrogen, while in a normal cheese of the same age the amount was 38.66 per ct. The enzymes present in this cheese were therefore able under favorable circumstances to accomplish about 72 per ct. as much decomposition of casein as occurred in a normal cheese. That they accomplish this fraction of the work under ordinary conditions does not necessarily follow. These results show merely that the peculiar conditions of manufacture in the presence of chloroform were not such as to prevent the enzymes from rendering cheese-casein soluble.

INFLUENCE OF SMALL AMOUNTS OF ACID ON ENZYME ACTION.

In the ordinary process of manufacture there is a gradual formation of acid within the mass through the action of bacteria. In the preceding experiment acid was necessarily absent. To remedy this, another cheese was made like the preceding except that lactic acid was added.

As before, 3.5 lbs. of chloroform were added to 125 lbs. of night's and morning's milk, sufficiently acid for cheese-making. This was curdled by one-quarter ounce of Hansen's liquid rennet added at 86° F. (30° C.). After cutting the curd and applying heat, pure lactic acid was added in small quantities at a time until the whole amounted to nearly .2 per ct. of the milk used. One-half of the resulting curd, unsalted, was pressed into a Young America cheese which, fresh from the press, contained 32 per ct. of water and 15 per ct. of chloroform.

The results of the examinations are shown below:

TABLE VII.—COMPARISON OF CHLOROFORM CHEESES MADE WITH AND WITHOUT LACTIC ACID.

Conditions of making and curing curing.	Total water-soluble N. formed for 100 lbs. N. in cheese.					
	1 month.	2 months.	3 months.	6 months.	9 months.	12 months.
Cheese No. 30 A. made and cured with chloroform.	5.70	8.20	11.60	14.50	19.50	22.60
Cheese No. 32A. made and cured with chloroform and lactic acid.....	5.70	9.40	14.00	20.60	23.20	31.65

It will be seen that in cheese 32A the amount of soluble nitrogen is greater than in 30A after the first month and continues to become greater up to the end of 12 months, the age of 32A at its last analysis. This more rapid ripening in 32A took place in spite of the fact that only one-half as much rennet was used in 32A as in 30A. Acid appears to favor enzyme action.

INFLUENCE OF SALT UPON ENZYME ACTION.

In the two preceding experiments it has been noted that one-half the curd was pressed without salting and the results previously given represent the changes taking place in unsalted cheese. However, in the manufacture of Cheddar cheese, salt is never omitted and, in order to make the comparison between the chloroform cheese and normal cheese complete, the addition of salt is required.

In each of the two experiments, one-half of the curd was salted just before putting to press, the first receiving 2 ounces and the second 2½ ounces. In each case the percentage of chloroform and water was essentially the same as in the corresponding unsalted portion. The results of analysis are shown in the following table. To facilitate comparison, the results from the unsalted portions are also repeated.

TABLE VIII.—COMPARISON OF CHEESES MADE AND CURED WITH CHLOROFORM, SALTED AND UNSALTED.

Conditions of curing.	Total water-soluble N. formed for 100 lbs. N. in cheese						
	1 mo.	2 mos.	3 mos.	6 mos.	9 mos.	12 mos.	15 mos.
(1) Without lactic acid :							
Cheese No. 30A, made and cured with chloroform—not salted.....	5.70	8.20	11.60	14.50	19.50	22.60	27.70
Cheese No. 30B, same as 30-A, but salted.....	2.25	3.20	5.50	7.80	11.60	17.20	24.00
(2) With lactic acid :							
Cheese No. 32A, made and cured with chloroform and lactic acid—not salted.....	5.70	9.40	14.00	20.60	23.20	31.65	
Cheese No. 32B, same as 32-A, but salted.....	3.00	4.90	6.70	9.75	12.45	19.65	

From the results here given it is seen that salt in the proportion usually present in cheese exerts a strong repressing influence upon the activity of the enzymes present. On comparing this effect of salt in the case of the cheese containing added acid with the cheese in which acid was omitted, it is seen that acid favored enzyme action here also as well as in unsalted cheese.

The results of our work up to this time appear to show, (1) that the use of chloroform excludes bacterial action in milk and cheese and limits the work of ripening to those enzymes contained in milk when made into cheese; (2) that the presence of salt noticeably decreases the effect of such enzymes; (3) that the presence of two-tenths of one per ct. of lactic acid increases the ripening action, at least of rennet enzymes; (4) that the percentage of cheese-casein made soluble by the enzymes under consideration in nine months (which may be regarded as the extreme limit of the commercial life of Cheddar cheese, kept under usual conditions) is about 12 per ct., or one-third the amount of soluble nitrogen found in normal cheese; and (5) that the amount of ripening caused by enzymes present in the milk when made into cheese is apparently more limited than was previously supposed.

We may say that the limited part apparently taken by such enzymes in ripening cheese is a result we did not anticipate when undertaking the work. We have additional experimental work under way for the purpose of testing these results more rigidly.

DIFFERENCE IN CHARACTER OF CHEMICAL CHANGES IN NORMAL AND IN CHLOROFORM CHEESE.

An examination of the detailed data secured with normal and with chloroform cheese shows clearly a marked difference in the character of the changes taking place in the soluble nitrogen-compounds. This difference is seen if we study the amounts of albumoses and peptones in relation to amides, and also the relative amounts of ammonia found.

The following tabulated comparison in case of cheeses 34C and 34B, which were made with and without chloroform from different portions of the same milk, illustrate the points in question.

TABLE IX.—SHOWING DIFFERENCE IN CHARACTER OF CHEMICAL CHANGES IN NORMAL AND IN CHLOROFORM CHEESE.

Character of cheese.	Age.	N. in albumoses and peptones.	N. in amides.	Ratio of (1) to (2).	N. in ammonia.
	Months.	(1)	(2)		
Cheese 34 C—normal.....	1	2.95	5.42	1:1.80	0.86
" 34 B—chloroform.....	1	3.71	0.86	1:0.23	0
Normal cheese.....	1½	2.51	8.49	1:3.40	1.29
Chloroform "	1½	7.31	1.82	1:0.25	0
Normal "	3½	5.37	12.60	1:2.40	2.51
Chloroform "	3½	10.20	3.22	1:0.31	0
Normal "	5½	4.97	18.50	1:3.70	3.38
Chloroform "	5½	12.40	4.73	1:0.39	0
Normal "	7	3.08	20.10	1:6.50	4.42
Chloroform "	7	10.90	8.11	1:0.74	0
Normal "	9	2.70	23.50	1:8.70	4.87
Chloroform "	9	12.52	11.60	1:0.93	0

Stated in a general way, these results show (1) that, in cheese made and cured with chloroform, the amount of albumoses and peptones is largely in excess of the amount of amides; (2) that the reverse is true in normal cheese; and (3) that ammonia appears in normal cheese much earlier and in larger quantities than in chloroform cheese.

Making a detailed comparison, we note the following points:

(1) In the normal cheese at the age of one month, the amount of amides was 1.8 lbs. for each pound of albumoses and peptones. This ratio increased until at nine months it was 8.7, nearly five times as great as at the end of one month.

(2) In the chloroform cheese, the amount of amides was not quite one-fourth of the amount of albumoses and peptones at the age of one month. The relative amount slowly increased, until at the end of nine months the amount of amides was nearly equal to that of albumoses and peptones,

(3) In the chloroform cheese, no ammonia had appeared at the end of nine months; in the normal cheese, nearly one per ct. of the total nitrogen was present as ammonia at the end of one month and this amount steadily increased.

From these results it is seen that, in a normal cheese, the amides steadily increase, while the albumoses and peptones

increase for some months and then decrease. In a chloroform cheese, the different classes of compounds under discussion all increase continuously from the beginning for many months.

In normal cheese, traces of ammonia appear at an early stage of ripening, while, in chloroform cheese, the first traces usually appear only after the lapse of six months or more, and the increase is very slow, so that even after a year only minute amounts are present.

From these data it appears that there is some agent at work in normal cheese which is not active in cheese made with chloroform. Just what this additional factor is our present data do not explain, but our efforts are being directed to the task of identifying this agent.