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REPORT

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Committee on Thesis

The undersigned, acting as a Committee of the Graduate School, have read the accompanying thesis submitted by A. C. Dahlberg for the degree of Master of Science They approve it as a thesis meeting the requirements of the Graduate School of the University of Minnesota, and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science

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THE ENZYMES OF BUTTER

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A THESIS

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BY

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INTRODUCTION AND HISTORICAL.

The deterioration of butter during storage is often attributed to the enzymes in the buttermilk it contains. Most experimental work on this subject has only an indirect bearing since it deals with the chemical changes taking place in butter on standing and the exclusion of microorganisms as a probable cause.

Rahn, Brown, and Smith [1] stated that "all (storage) butter investigated showed an increase of "amid nitrogen", i.e. nitrogen not precipitated by copper sulphate, tannic acid, or phosphotungstic acid." Their methods were shown to be at fault by Rogers, Berg, Potteiger, and Davis [2] who could observe "no evidence of an increase in soluble nitrogen in butter on long standing at 0°F., even when the conditions of manufacture were most favorable to such changes." They could detect no proteolysis in buttermilk held a long period of time in cold storage to which 18% sodium chloride had been added, but active bacterial proteases, pepsin, and trypsin were not completely inhibited in their action by these adverse conditions. Lactose, they concluded, was oxidized only when a trace of iron and peroxide were added. Rogers [3] found lipase to be the cause of the increase in the acidity of canned butter on standing.

During the summer of 1915, the winter, as a part of his duties in connection with the Division of Dairy and Animal Husbandry of the Minnesota Agricultural Experiment Station, prepared a number of lots of butter and placed them in storage, in order to study the effect of varying conditions of manufacture and storage upon the keeping qualities of the butter. These afforded an excellent opportunity for a study of the enzyme content of the butter after storage and such a study was accordingly undertaken.

The work reported in this paper shows in what relative quantities the enzymes of the milk are found in the butter, the influence of storage on their

strength, and, incidentally some of the factors influencing the rate of galactase activity.

EXPERIMENTAL WORK

Investigations on Galactase.

1. Length of the Period of Action. Galactase, so named by Babcock and Russel [6], is a normal proteolytic enzyme of milk characterized by its slow decomposition of the milk proteins into peptones, amino acids, and ammonia.

Experimental data gives quantitative proteolytic acitvity for periods varying from an hour for certain pepsin work [4] to the prohibitive time of one thousand five hundred and thirty-six days for galactase [5]. The time which most investigators have allowed galactase to act to obtain comparative results ranges from thirty days to a year. It appears that this is unnecessarily long. The analytical data of Babcock and Russel [6] gives an average increase in the soluble nitrogen content of a number of samples of skimmilk, when expressed as percent of the total nitrogen, of 13.49% for the first eight days and only 5.66% for the following twelve days. One sample of skimmilk increased 26% in soluble nitrogen during the first seven days, 2% the following seven days, and to increase the soluble nitrogen content another 26% required a period of one hundred and eleven days. There is very little difference in the comparative galactase content of various skimmilks, when analyses at the end of seven days or six months are compared. In the present work the period of action was limited to four days because the soluble nitrogen in skimmilk increased 10 or 12% during this time.

2. Measurement by the Ninhydrin Method. The ninhydrin method of Harding and MacLean [7] for determining amino acid alpha nitrogen was compared with the Official Method of the Association of Official Agricultural Chemistry [8] for determining an increase in soluble nitrogen. In the ninhydrin determination the sample was diluted 1 to 10 and 1 cc. of this used in each analysis, but the results were calculated to the 10 cc. basis. Bacterial action was inhibited by 0.5% chloroform. All samples in all the work reported in this paper were incu-

bated at 40°c. for four days.

	Boiled Skimmilk	Skimmilk 3% Nacl	Skimmilk
Total nitrogen	.0586	.0586	.0586
Initial soluble nitrogen	.0033	.0108	.0108
Initial ninhydrin nitrogen	.0029	.0029	.0029
48 hr. soluble nitrogen	.0039	.0137	.0177
48 hr. ninhydrin nitrogen	.0032	.0031	.0032
% increase in soluble nitrogen	1.02	4.95	11.77
% increase in ninhydrin nitrogen	.51	.34	.51

The ninhydrin method indicated no increase in nitrogen when there was an actual increase of 11½% in the soluble nitrogen. This was further substantial by work on a pure casein solution which gave only a trace of nitrogen by the ninhydrin method with no increase after evident proteolysis had taken place. This method of estimating the rate of hydrolysis of proteins was, therefore, abandoned as not being applicable to investigations with milk proteins.

3. The Influence of Chloroform on Proteolysis. To prevent bacterial action in the samples chloroform was added in amounts found ample by Harding and VanSlyke [9]. Skimmilk to be analyzed at varying intervals extending over a long period of time was anaesthetized by 1% chloroform which, according to the above investigators and Babcock and Russel [6], should have only slightly inhibited proteolysis. As shown in the following table, the soluble nitrogen of this skimilk did not increase on standing. In verifying this result the soluble nitrogen was obtained by a Kjeldahl determination of the nitrogen in the total filtrate from the casein precipitation. Closer agreement in the duplicates could be obtained from the filtrates than from the casein itself.

Sample	%Chloroform	Total nitrogen	Initial soluble nitrogen	Soluble nitrogen after 4days	Increase in soluble nitrogen as % of total
1. Skimmilk	.5	.0583	.0129	.0183	9.26
"	1.0	.0583	.0130	.0114	-2.74
2. Skimmilk	.5	.0531	.0115	.0170	10.35
"	1.0	.0531	.0115	.0120	.94
3. Skimmilk	.5	.0406	.0119	.0136	4.18
"	1.0	.0406	.0119	.0103	-3.94
4. Skimmilk	1.0	.0547	.0106	.0079	-4.93
5. Buttermilk, neutral	.5	.0551	.0125	.0201	13.60
"	1.0	.0551	.0125	.0174	9.80
6. Buttermilk, neutral	1.0	.0463	.0064	.0059	-1.08

Sample No. 5 was buttermilk obtained from a small hand churning and probably contained 1% butterfat. Since fat destroys the anaesthetic property of chloroform by combining with it the results of No. 5 should not be considered. In every other case 1% of chloroform in skimmilk or buttermilk completely inhibited proteolysis.

4. The Influence of Sodium Chloride in Proteolysis. The influence of sodium chloride on the galactase activity of skimmilk emphasizes the necessity of removing all the salt from the butter extracts. A complete precipitation of the casein in the presence of 15 to 20% of common salt was obtained by diluting the sample with 150 cc of distilled water at 42° C and adding 10% acetic acid until no more precipitate formed. In some determinations more casein was precipitated by further dilution of the filtrate with the wash-water from the precipitate in which case refiltering was necessary. The samples were preserved by .75% chloroform.

Percent salt	Total nitrogen	Initial soluble nitrogen	Soluble nitrogen after 21 days.	Soluble nitrogen after 72 days.	Percent increase
0,boiled	.0561	.0055	.0051	.0034	-3.74
0	.0561	.0121	.0188	.0198	13.72
.5	.0561	.0121	-	.0171	8.91
1.0	.0561	.0121	.0142	.0131	1.78
15.0	.0534	.0121	-	.0110	- 2.06
20.0	.0528	.0121	.0120	.0103	- 3.40

The effect of sodium chloride is marked; both 15 and 20%, the concentration of the salt brine in butter, stopped all proteolysis. The very slight increase in the soluble nitrogen content of the skimmilk containing 1% salt is partially caused by the chloroform as the following work, in which .5% of the anaesthetic was used, will show.

Percent salt	Total nitrogen	Initial soluble nitrogen	Soluble nitrogen after 5 days.	Percent increase
0, boiled	.0586	.0033	.0034	.51
0	.0586	.0108	.0242	22.86
.8	.0586	.0108	.0201	15.87
3.0	.0586	.0108	.0143	5.97

The actual checking of the soluble nitrogen increase due to 1% of sodium chloride was approximately 10% in either case, but 3% of salt did not entirely prevent proteolysis.

5. Tracing Galactase from Milk to Butter. In following the protease of the fresh milk to the finished the reaction of the various products was brought to that of the fresh milk, i.e. 10cc were exactly neutralized by 1.5cc of n/10 alkali when phenolphthalein was used as an indicator, by dipping a stick of sodium hydroxide into the sample. If the sample became too alkaline it was neutralized by some of the original sample so dilution was avoided. The various samples were treated with chloroform in the following proportions: the skimmilk, buttermilk, and bowl contents, 0.5%; the milk, 2.5%; and the cream which contained

23% butterfat, 5%. The "bowl contents" was an emulsion of the ~~skime~~ slime in the wash-water held in the bowl. An equal volume of boiled skimmilk was added as a substrate, but the increase in the soluble nitrogen was calculated on the basis of the bowl contents alone. The "cream during ripening" refers to the proteolysis occuring during the ten hours it was ripened at 85°F. and the twenty hours it was held at 54°F. previous to churning.

Sample	Total nitrogen	Initial soluble	4 day soluble	percent increase	Initial percent of total nitrogen as casein.
Skimmilk	.0583	.0129	.0183	11.10	77.87
Whole milk	.0544	.0113	.0198	15.62	79.23
Cream	.0403	.0076	.0142	16.37	81.14
Bowl contents	.0189	.0046	.0262	114.28	89.93
Cream during ripening	.0403	.0076	.0081	1.66	
Cream after ripening	.0403	.0081	.0139	14.39	
Buttermilk	.0551	.0125	.0201	13.60	

In separating milk galactase is taken out of the skimmilk part, slightly increased in the cream, and highly concentrated in the separator slime. While no relationship exists between the increase in soluble nitrogen and the total nitrogen it is evident that the factors at work during milk separation which increase the percent of casein in the total nitrogen also increase the galactase content. The cream underwent a slight preoteolytic digestion during the ripening process, but the proteolysis after souring and neutralization was less than that of the sweet cream. This indicates that the chief proteolytic enzyme of milk is not of bacterial origin as Olson [5] recently contended.

6. Amount of Galactase Contained in Butter. The butter used in the experiments represented both good and bad qualities. The "fresh dairy" butter was made from the cream obtained from the milk of the University Farm herd, and was soured spontaneously without pasteurization. The "fresh creamery" butter was made in a cooperative creamery from sweet pasteurized cream ripened by a

commercial starter. Both of these butters were of extra quality. The "stored dairy" butter was the same as the fresh dairy excepting that it had been held in cold storage for eight months. The "fresh centralized" butter was made from the sour cream just as it was received by a central creamery. The last two butters mentioned were of poor quality. The "stored centralized" butter was made in the same way as the fresh centralized but had been held eight months in cold storage. It was of extremely poor quality.

No special effort was made to obtain a pure enzyme extract from the butter . A known weight, usually four hundred grams, of butter was melted at 45°C in two long glass tubes about an inch in diameter. After the separation was complete the clear fat was hardened by immersing the tubes in cold water, and the curd solution then washed out. If an excess of fat remained in the extract it was removed by rewarming and centrifuging. This extract was then dialyzed at a temperature never exceeding 13°C in a parchment dialyzer until free of sodium chloride, the last six or eight hours of dialysis taking place in distilled water. This curd was made up to a given volume and used at once in the various tests.

The acetone-ether method of obtaining a fat-free, dry powder was also tried but left so high a percentage of common salt in the powder that dialysis was necessary. Consequently, this method had no advantages on the other method, and the enzymes probably would have been weakened by the precipitation.

The casein was precipitated by the Optional Official [8] rather than the Official method because filtering was often more rapid, the volume to be filtered much less, and a clear filtrate more easily obtained. Thirteen analyses of chloroformed skimmilk and butter curd extracts gave an average of .00104 grams more nitrogen in the form of casein by the use of alum as the precipitant, hence the methods should not be used interchangeably. A clear filtrate was more easi-

ly obtained and an excess of substrate assured by the addition of boiled skimmilk to the curd solutions. In a few cases the filtering had to be done on a "Buchner funnel" through three filter papers and repeated ten to twenty times to obtain a perfectly clear filtrate. Bacterial action was prevented by 1% chloroform instead of .5% because the extracts contained considerable fat. In the following table the column headed concentration first gives the ratio of butter to curd extract, the second that of curd extract to boiled skimmilk added.

Butter	Concentration.	Initial soluble nitrogen	Soluble nitrogen after 4 days.	Increase in grams	Increase in grams for 10 grams butter.
1.Fresh dairy	2/1, 50/100	.0098	.0108	.0010	.0015
Fresh dairy, boiled	2/1, 50/100	.0091	.0091	.0000	.0000
2.Fresh dairy	2/1, 50/100	.0080	.0090	.0000	.0015
Fresh dairy, boiled	2/1, 50/100	.0070	.0045	-.0025	.0000
3.Storeddairy	3/2, 50/50	.0043	.0063	.0020	.0027
4.Stored centralized	2/1, 50/50	.0042	.0078	.0036	.0036
Stored cent., boiled	2/1, 50/50	.0039	.0036	-.0003	.0000
5.Fresh creamery	3/2, 50/50	.0049	.0044	-.0005	.0000

The two samples of fresh dairy butter gave uniform and measurable proteolytic action while one sample of fresh pasteurized creamery butter showed no digestion of the casein. The increase in the soluble nitrogen of the stored butter was nearly twice that of the fresh butter.

The Lipase Content of Butter.

Lipase hydrolyzes fats into alcohols and fatty acids causing an increase in the acidity of the media acted upon. The increase in the acidity of butter on standing is due to this enzyme [3].

The curd solutions used in testing for lipolytic activity were just half the volume of the butter. The substrate was either 5% of butterfat or olive oil, and the preservative was 2.5% chloroform. Ten cc aliquots of the extracts in ten cc of neutral 95% alcohol were titrated against n/40 sodium hydroxide using phenolphthalein as indicator.

Sample	Initial	4 days	Increase over boiled.
1. Fresh dairy, boiled	.2	1.3	0
Fresh dairy	.2	1.1	0
None	.1	.8	0
2. Fresh unsalted dairy, boiled	5.3	5.3	0
Fresh unsalted dairy	6.3	6.6	.3
3. Stored unsalted dairy	5.5	5.5	0
4. Fresh centralized	2.7	3.0	.3
5. Stored centralized	2.4	3.5	1.1
6. Fresh creamery	1.8	2.2	.4

The extracts from the unsalted butters were not dialyzed so the initial acidity was high. Lipolytic action at the end of four days at 40°C was too small to be measured with the exception of the stored centralized butter.

The Oxidase Content of Milk and Butter.

Since the investigations of oxidase acitvity in milk have been limited to a few easily oxidized substances, and since oxidases show a specificity towards chemicals in their action it was thought desirable to try the action of milk on the common chromogens by Bunzel's method [10]. Four cubic centimeters of milk were used in each test. Negative results were obtained with paraphenylene diamine, phenolphthalin, hydrochinon, pyrocatechin, phloroglucen, alpha naphthol, and para, meta, and ortho cresols. Positive results were obtained with metol and pyrogallol but since the action of the latter was about 20% the greater pyrogallol alone was used for the final determinations. The results tabulated below were obtained in duplicate, the duplicates agreeing very closely. The readings are given in millimeters of mercury, each millimeter representing an absorption of .025 cc. of oxygen.

Time in Minutes	Skimmilk	Whole milk		Skimmilk	
		Raw	Boiled	Raw	Boiled
60	-	.16	.00	.00	.00
80	-	.40	.00	.00	.00
100	-	.56	.00	.17	.00
120	.95	.70	.16	.17	.00
140	1.07	.85	.20	.35	.00
160	1.28	.97	.27	.40	.00
180	1.33	1.13	.40	-	-
300	-	1.70	.91	-	-
360	-	1.97	1.22	-	-
420	-	2.25	1.48	-	-
460	-	2.32	1.55	-	-

There was no agreement in the results obtained from the skimmilk from the same herd on different days, boiling failed to completely inhibit the activity of the whole milk, and the action was very slow in starting. It cannot be caused by a normal milk oxidase. In every case a browning of the samples was quite marked before oxygen absorption started.

Seven curd solutions from different butters gave no action with pyrogallol.

The Catalase Content of Milk and Butter.

The method of measuring the catalase of milk given by Barthel [11] gave very closely agreeing duplicates and was used in this work. In milk the liberation of oxygen ceased in four hours. The curd extracts were equal in volume to the original butter. Five percent of hydrogen peroxide was quantitatively added to the samples to be tested and the grams of peroxide in the boiled samples at the end of four hours were used as the basis for calculation.

Sample	H_2O_2 Content after four hours.	Grams H_2O_2 less than in boiled.
1. Boiled milk	.0054	.0000
Milk	.0039	.0015
2. Boiled fresh dairy	.0054	.0000
Fresh dairy	.0049	.0005
3. Stored dairy	.0049	.0005
4. Fresh centralized	.0026	.0028
5. Stored centralized	.0039	.0015
6. Fresh Past. creamery	.0048	.0006

The fresh centralized butter did not contain as much catalase as the results show because a clear filtrate was not obtained. Every sample of butter contained catalase. That the centralized butter gave higher results than the other butter and as high as milk itself agrees with the belief that there is some relationship existing between catalase activity in milk and bacterial growth. Storage did not diminish its strength.

The Peroxidase Content of Milk and Butter.

The common Starch test for heated milk was used on the butter extracts withe the following results:

Sample	Time in Minutes				
	0	5	10	20	30
1. Fresh dairy, boiled	white	white	white	white	white
Fresh dairy	"	gray	gray	gray	gray
2. Stored dairy	"	white	white	faint gray	faint gray
3. Fresh centralized	"	gray	gray	gray	gray
4. Stored centralized	"	white	faint gray	faint gray	faint gray
5. Fresh past.creamery	"	white	white	faint gray	faint gray

All the samples gave some color change but for the storage butters and the pasteurized creamery butter it was very slight. Milk diluted 1 to 160 with distilled water and to which 16% boiled skimmilk was added gave a gray color similar to that of the fresh dairy butter. Hence, the peroxidase content of butter is very small.

A Comparison of Milk and Butter Enzymes on the Basis of Total Nitrogen.

The fat in butter acts as so great a dilutant for the water soluble constituents that a direct comparison of the enzymes of milk and butter is misleading. The more logical comparison is on the basis of the total nitrogen since the proteins and enzymes exist in the same colloidal state and ought to be carried into the butter in the same proportions as they exist in the cream. The total protein of the fresh dairy butter was .55%, that of the milk from which it was made 3.47% so the enzymic activities in the butter were multiplied by 6.31. Taking the milk as the standard and its enzymic content as 1 butter was found to contain the following amounts:

Galactase	1.1
Oxidase	0.0
Catalase	2.0
Peroxidase	.008

The galactase content of the butter was equal to that of the milk, the catalase content was double, but the peroxidase content was very much less.

SUMMARY.

The nitrogen in skimmilk indicated by a ninhydrin determination was not influenced by an increase in the soluble nitrogen.

Proteolysis in skimmilk was completely inhibited by 1% of chloroform and by 15% sodium chloride. Galactase cannot act in normal butter because of the high salt content.

In the separation of milk the factors which increase the percent of casein in the total nitrogen also increased the galactase content. The ripening of cream did not increase the rate of proteolysis.

No oxidase was found in milk or butter.

Only one sample of butter gave any evidence of lipase at the end of four days at 40° C.

The enzyme content of butter is very small because of the high dilution in fat. Expressed on the basis of total nitrogen the butter examined contained as much galactase as fresh whole milk, twice as much catalase, but only 1/160 as much peroxidase.

The cold storage of butter weakens the peroxidase but has little effect on the catalase and galactase.

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