UNIVERSITY OF MISSOURI COLLEGE OF AGRICULTURE
AGRICULTURAL EXPERIMENT STATION
RESEARCH BULLETIN 34

THE PRESERVATION OF MILK FOR CHEMICAL ANALYSIS

(Publication Authorized November 10, 1918)

COLUMBIA, MISSOURI
JUNE, 1919

UNIVERSITY OF MISSOURI

COLLEGE OF AGRICULTURE

Agricultural Experiment Station

THE PRESERVATION OF MILK FOR CHEMICAL ANALYSIS

L. S. PALMER

With the technical assistance of L. H. Cooledge

INTRODUCTION

It is not always feasible for the chemist engaged in the analysis of milk to make all the determinations on the fresh sample. It is customary in such cases to add small quantities of various preservatives, such as formaldehyde, mercuric chloride etc., in order to prevent the fermentation and decomposition which milk undergoes on standing. Some study has been made of the proper quantity of these preservatives to add for ordinary preservation for short periods of time and the results have led to the adoption of what may be called standard amounts of these substances. For example, the official methods of the A.O.A.C.[1]* recommend one part of formaldehyde to 2,500 parts of milk.

The problem of the preservation of milk for chemical analysis becomes more difficult, however, when it is necessary not only to prevent the common fermentations to which milk is subject but also to preserve the individual constituents of the milk unchanged for considerable periods of time. The need has long been felt for an efficient preservation of this kind. Unfortunately, our knowledge of the changes which the various milk constituents undergo under the influence of preservatives has been limited, so that the selection of any one or more preservatives which will give an efficient preservation of the several milk constituents has not been possible.

It was the purpose of the investigation reported in this bulletin to give the subject of milk preservation a more thoro study than it has hitherto been accorded. It was hoped that the results would lead to the selection of some method of milk preservation which would be more efficient from the standpoint of the individual constituents than the methods heretofore available.

*This and subsequent numerical references are to the Bibliography.

HISTORICAL[2]

The preservatives selected for comparison in this study were formaldehyde, mercuric chloride, potassium dichromate, copper sulfate, thymol, and toluene. Most of these preservatives have been the subject of more or less study at various times and a brief resume of what has been done along this line will, therefore, not be out of place.

Formaldehyde.—Price[3] found that formaldehyde in the proportion 1:1560, after twenty-four hours incubation, had destroyed the common bacteria found in milk but that it required an incubation of seventy-two hours to destroy them in the presence of formaldehyde 1:1870. Rivas[4] found that formaldehyde 1:1000 killed all the bacteria in certain milk samples. Seligman[5] found that formaldehyde 1:5000 exerted a selective action on the bacteria of milk, preventing the development of lactic acid forming bacteria to a greater extent than other kinds. Schaps[6] found that Staphylococcus pyogenes was more resistant than lactic acid bacteria to formaldehyde 1:5000 to 1:10000. Sommerfeld,[7] studying the germicidal power of very dilute solutions of formaldehyde, i. e., 1:12,500 to 1:25,000, at various temperatures, found them to be effective at 10-15°C., only, or lower. At 37°C. there was no appreciable action of such dilute solutions of formaldehyde.

A number of investigators have made a study of the action of various enzymes of milk in the presence of formaldehyde, with the general result that their action has been bound to be greatly retarded and in some cases, perhaps, entirely inhibited. The only exception recorded is that of the oxidizing enzymes which Seligman[5] found to be accelerated by the addition of formaldehyde 1:12,500.

The action of formaldehyde upon the natural proteoclastic enzyme of milk has been the subject of some study and is of special interest in connection with the preservation of milk for chemical analysis. Babcock, Russell, and Vivian,[8] who named the proteoclastic enzyme of milk galactase, found that formaldehyde considerably weakened its activity. Freudenreich[9] also found that one per cent formalin greatly retarded the proteolysis in skimmilk kept at 35°C. for six weeks, but did not prevent it entirely. Jensen,[10] studying the activity of galactase in the presence of formalin at both room temperature and 35°C., found that there was no greater activity at the higher temperature in comparison with the lower, "probably because formalin, like other similar antiseptics, ex-

erts a much stronger influence at higher temperatures than at lower temperatures." Van Slyke[11] found that formaldehyde 1:2,500 greatly retarded the activity of the natural galactase of milk, especially in comparison with chloroform, when the milk was kept at 37°C., but did not prevent entirely the increase in soluble nitrogen. Tice and Sherman[12] made a study of the proteolytic changes in samples of milk kept at room temperature for eleven to forty-three months after the addition of formaldehyde in the proportions of 1:1000 to 1:1400, and found that the addition of the preservative had merely retarded the proteolysis. Price,[3] studying the action of formaldehyde on the activity of digestive enzymes *in vitro*, found that formaldehyde 1:20,000 did not interfere with the action of galactase. He investigated two samples of milk containing formaldehyde 1:1000 and 1:1400, respectively, and found that when kept for two weeks at 40°C. there was no increase in the soluble nitrogen. He found also that adding formaldehyde in the proportion of 1:1500 to milk containing a concentrated extract of galactase in addition to its own natural enzyme content, retarded the activity of the galactase about 85 per cent but did not prevent it entirely.

There is some evidence to support the view that the retarding action of formaldehyde is due to some action which it exerts upon the proteins rather than to any actual inhibiting of the action of the enzymes. For example, a number of investigators[13] have studied the retarding action of formaldehyde on the coagulation of milk by the rennet enzyme, and most of them agree with the view that this phenomenon is due to some action upon the casein of the milk. Cochran,[14] especially, has shown that formaldehyde has a marked hardening action on casein, increasing with the length of time the formaldehyde is in contact and causing the casein to be much less soluble in concentrated sulphuric acid.

Formaldehyde forms methylene derivatives with free amino groups. Presumably this reaction takes place with all the free amino groups in a protein molecule when sufficient formaldehyde is present, but it is difficult to understand why this should interfere with the hydrolysis of the protein molecule which occurs at the peptid linkages. An explanation of the retarding action of formaldehyde on hydrolytic decompositions accelerated by enzymes must accordingly be sought in other directions, possibly in connection with an action upon some of the physico-chemical properties of either the proteins or the enzymes themselves, inasmuch as these properties of both proteins and enzymes are believed to be intimately connected with their reaction with one another.

Formaldehyde has been found to exert a detrimental influence upon certain of the properties of proteins. Thus Blum[14] and Bach[16] have found that both egg and serum albumin lose their coagulability by heat on treatment with formaldehyde. Lactalbumin appears to be similarly affected, Cavanaugh[17] reporting that formaldehyde in certain concentrations considerably reduces the amount of lactalbumin which can be precipitated by heat.

With Cavanaugh's work as a basis, Patrick[18] and a number of others investigated the effect of various preservatives, including formaldehyde, upon the recovery of albumin from milk by heat coagulation. The result of their study of formaldehyde was that concentrations of the preservative between one part in 310 of milk and one part in 630 caused an apparent increase in the casein at the expense of the albumin when the milk had been allowed to stand for about three weeks. This phenomenon was not noticeable in three out of four samples to which formaldehyde had been added in the proportion of one part to 1660 of milk. With still smaller concentrations of formaldehyde, i. e., 1:2000 to 1.:2500, the samples showed evidence of proteolysis at the end of several weeks. The samples were all kept at room temperature in Patrick's investigation.

Mercuric chloride.—Very little work has been done on the effectiveness of this preservation, altho it is known to be a strong antiseptic.

Patrick,[18] in his study of milk preservatives, found that 2.5 to 5 grams of mercuric chloride per liter of milk acted very much like large amounts of formaldehyde, causing a marked increase in the casein nitrogen at the expense of the albumin. He secured no consistent results when a concentration of one gram per liter was used.

Tillmans, Splittgerber and Riffart,[19] comparing chloroform, thymol, mustard oil, phenol, creosote, sodium fluoride, potassium dichromate and mercuric chloride as milk preservatives found the latter only to be satisfactory. They recommend 0.03 to 0.04 per cent as the proper amount to use.

Gerber[20] studied the action of mercuric chloride toward both animal and vegetable rennets with the result that small amounts accelerated the rennet activity when added to the milk with the rennet. Large amounts, however, were found to retard the rennet activity.

Potassium dichromate.—This preservative is frequently used for milk, especially in European countries. For example, the French Government requires that it be used for milk samples which appear in legal cases.

Kühn[21] found that potassium dichromate is a better preservative at low than at high temperatures.

Patrick[18] has found, however, that this preservative does not prevent the decompostion of the casein and albumin of milk when used in as large amounts as 50 cc. of saturated solution per liter of milk.

Copper sulfate.—This preservative has never come into general use for milk altho it has been found to be strongly antiseptic toward certain mould spores. Gerber[20] has shown that it acts more unfavorably than mercuric chloride toward rennet.

The action of copper sulfate toward the milk proteins has never been studied.

Thymol.—This reagent has been used extensively as a preservative of feces and urine for chemical analysis, but very little mention is made in the literature of its use as a milk preservative. Richmond[22] states that "it keeps the milk, but allows the cream to rise in so firm a layer that it is not readily redistributed."

Seligman[5] found that thymol has no influence on the oxidizing enzymes of milk.

EXPERIMENTAL

In the various experiments reported in this bulletin attention was paid to the nitrogen distribution as represented by the casein, heat-coagulable protein, residual protein, and residual non-protein nitrogen. The total nitrogen was also determined in most cases. The percentage of fat and lactose was determined in a number of cases. In certain of the experiments tests for oxidase, peroxidase and catalase were performed, and bacterial counts made. The methods of analysis used were as follows:

METHODS OF ANALYSIS

Total nitrogen.—Five cc. of milk at 23°C. were pipetted into a Kjeldahl flask and digested with 25 cc. of concentrated H_2SO_4, 0.2 grams of $CuSO_4$, and 6.0 grams of K_2SO_4, the digestion being continued for 45 minutes after the mixture became clear. In titrating back the unused acid with NH_4OH solution after distillation, Congo Red was used as indicator.

Casein.—Ten cc. of milk were pipetted into a clean beaker and warm water added until the temperature of the diluted milk was 44-46°C., the addition being adjusted so that the final volume was

about 140 cc. Approximately 14 cc. of 1.5 per cent acetic acid solution were then added from a burette with constant stirring. After settling, the precipitate was filtered off using a Munktell No. O. B. paper. The precipitate was washed with 0.15 per cent acetic acid, and the nitrogen determined upon the filter paper and precipitate as in the case of the total nitrogen.

Heat-coagulable protein.—The filtrate from the casein, together with the washings, which usually amounted to about 225 cc., was neutralized with dilute NaOH solution, using phenolphthalein as indicator, and 2 cc. of 1.5 per cent acetic acid solution added. The solution was then quickly raised to the boiling point and boiled for four minutes. The precipitate was filtered off and treated as in the case of the casein determination with the exception that it was washed with water only.

Residual protein.—Fifteen cc. of Almen's[28] tannic acid solution were added to the filtrate and washings from the heat coagulum. The precipitate, after standing a short time, was filtered off, washed with water, and the nitrogen determined as in the case of the heat-coagulable protein.

Residual non-protein nitrogen.—The filtrate and washings from the residual protein were rinsed into a Kjeldahl flask and the nitrogen content determined as in the case of the total nitrogen.

Fat.—Except where stated the fat was determined by the official Babcock asbestos method of the A.O.A.C., weighed portions of the whole milk being absorbed by asbestos in perforated copper cylinders, dried to constant weight in a steam oven, and extracted with ether for 24 hours, dried to constant weight again and the fat calculated from the loss in weight on extraction. In certain cases the fat was determined by the Babcock centrifugal method. Whenever this method was used it is so stated in connection with the data.

Lactose.—The lactose was determined by the official optical method of the A.O.A.C., using mercuric nitrate as the precipitant.

Bacterial counts.—Bacterial counts were made by the standard methods of the American Public Health Association for water analysis, using beef extract, however, instead of beef infusion. Two per cent of lactose was added for the lactose agar and lactose gelatin. The reaction of all media was brought to plus 1.0 per cent. The gelatin plates were incubated for three to five days at 20°C., and the agar plates for 24 to 36 hours at 38°C.

Oxidase and peroxidase tests.—All the enzyme tests were

taken from the work of Rogers, Berg and Davis[24]. Oxidase and peroxidase were determined by placing 10 cc. of the milk in a test tube and allowing two to three drops of a freshly prepared, ten per cent, alcoholic solution of gum guaiac to run down the side of the tube so that the tincture remained on the upper surface of the milk. The tube was allowed to stand for five to ten minutes. The failure of a blue color to appear was taken to indicate the absence of oxidase. Two or three drops of dilute hydrogen peroxide solution were then added to the test tube. If peroxidase was present a blue color developed where the reagents came in contact. Usually a blue ring formed on or near the surface.

Catalase.—Fifty cc. of milk were introduced into a 100 cc. Erlenmeyer flask and 25 cc. of commercial hydrogen peroxide added. The flask was quickly closed with a stopper provided with a bent glass tube, the other end of which was then inserted into a fermentation tube (capacity 10 cc.) filled with water. The oxygen liberated by the catalase was caught in the tube and the amount liberated in fifteen minutes recorded. The reaction was practically complete in this time. The results are expressed as the percentage of the 10 cc. volume filled by the gas in the given time.

PRELIMINARY EXPERIMENTS

As a preliminary study a number of trials were made using formaldehyde, mercuric chloride, potassium dichromate, copper sulfate, thymol and toluene as preservatives, in which the preservatives were compared under like conditions. Several trials were made with each preservative, using various amounts. In these experiments attention was paid to the nitrogen distribution of the milk only. In reporting the results the experiment which showed the most efficient preservation in each case is given. These data are presented in Table 1. They represent the average of at least two, and in some cases several, concordant figures.

In presenting these data the heat-coagulable and residual protein nitrogen are shown separately and are also shown together in a separate column under the heading, total albumin and globulin. This procedure is followed thruout the entire bulletin.

At the time the data presented in this bulletin were taken the view was generally held that the relative proportion of non-heat-coagulable protein in milk, as determined by a reagent such as tannic acid, gave a measure of the decomposition of casein and albumin to

TABLE 1.—COMPARISON OF HCHO, $K_2Cr_2O_7$, $HgCl_2$, $CuSO_4$, THYMOL AND TOLUENE AS PRESERVATIVES OF MILK PROTEINS

Preservative	Concentration of preservative	Temperature of preservation	Time of analysis	Casein N.	Heat-Coag. N.	Residual proteinN.	Total albumin and globulin N.	Residual non-prot. N.	Total N.
		°C	days	per cent	per cent	per cent	per cent	per cent	per cent
Fresh	0	....	1	0.357	0.061	0.041	0.102	0.028	0.498
HCHO	1:2,500	8	29	.351	.034	.058	.092	.053	.498
Fresh	0	....	1	.379	.055	.038	.093	.028	.498
$K_2Cr_2O_7$	1:200	8	31	.356	.031	.074	.105	.028	.498
Fresh	0	....	1	.357	.061	.049	.110	.028	.498
$HgCl_2$	1:1,000	8	29	.359	.060	.033	.093	.050	.498
Fresh	0	....	1	.379	.....	.....	.....	.....	.....
$CuSO_4$	1:350	8	35	.379	.....	.....	.....	.....	.....
Fresh	0	....	1	.417	.056	.038	.094	.028	.533
Thymol	1:5,000	2	17	.394	.074	.049	.123	.028	.533
Fresh	0	....	1	.407	.056	.038	.094	.028	.533
Toluene	1:1,000	2	17	.392	.069	.050	.119	.028	.533

secondary proteins. Shortly after the completion of the experimental work certain experiments which the author carried out regarding the quantitative analysis of the albumin and globulin of milk threw considerable doubt upon the correctness of the interpretation of the data from this point of view.

In explanation of the delay in publishing these data it may be said that the author has now become convinced, as the result of much study and experimental work, that the distribution of albumin and globulin between coagulable and non-coagulable portions is purely an arbitrary matter, and that the circumstances which determine which fraction shall be the larger and which the smaller are largely fortuitous, as far as our present knowledge is concerned. The author no longer holds the view as tenable that tannic acid gives a measure of secondary proteins in milk after the albumin has been removed by heat coagulation. Fresh milk shows a high proportion of such secondary proteins even when heat-coagulation is carried out under the most carefully controlled conditions and by the most approved methods.

It does not seem to be generally recognized that there has never been any proof offered that fresh milk contains secondary proteins, except possibly in the merest traces. It is evident, therefore, that heat coagulation does not give an accurate measure of any protein constituent of milk, but is merely an arbitrary method subject

to wide variations, depending upon the kind of preservative used, the acidity and concentration of the solution, the length of time the solution is boiled, and other conditions. These facts are abundantly supported by much of the data given in this bulletin. According to the notion that heat-coagulation gives a measure of the albumin, there were very few of the experiments in which the "albumin" was not subject to extensive proteolysis on standing, in some cases for a very short time only, in contact with certain preservatives.

The variations between heat-coagulable and residual protein have therefore been disregarded in drawing conclusions in regard to the efficiency of the preservatives studied. The data are presented, however, in the exact form in which they were taken, but they are interpreted on the basis of variations in the casein, total albumin and globulin, and residual non-protein nitrogen.

It is recognized that this method is subject to the error that it gives no measure of any secondary proteins which have formed as the result of proteolysis, inasmuch as tannic acid causes a quantitative precipitation of most secondary proteins. However, since no practical method has, as yet, been devised for separating primary from secondary proteins quantitatively, it has been necessary to depend upon changes in the casein, and accompanying changes in the albumin and globulin and non-protein nitrogen for evidence of proteolytic changes in the milk. A marked increase in non-protein nitrogen at the expense of the other nitrogenous constituents would also justify the conclusion that proteolysis had taken place.

In the preliminary investigations, the principal results from which are shown in Table 1, formaldehyde appears to have been the most efficient preservative, altho there was some proteolysis of the albumin and globulin even in this case. Mercuric chloride apparently preserved the casein unchanged for 29 days when added in the proportion of one part to 1000 of milk. Potassium dichromate, thymol and toluene did not prove to be effective as preservatives of the protein constituents under the conditions studied. Proteolysis was so marked even under the best conditions that a future study of these preservatives was abandoned. The single brief test with copper sulfate, however, indicated that it deserved a much more thoro study.

One very important feature of the results of the preliminary studies was to point out the probable importance of factors other than the amount of preservative upon the efficient preservation of the milk. A second series of experiments was therefore undertaken

for the purpose of determining the importance of a number of secondary factors such as the temperature of preservation, the initial bacterial condition of the milk and the amount of air in contact with the sample. In nearly all of these studies the analyses were extended to include the percentage of fat and lactose in the milk.

INFLUENCE OF TEMPERATURE ON PRESERVATION

The object of this experiment was to study the influence of the temperature of preservation with four different preservatives. The temperatures studied were 2°C. and 14°C. The preservatives used were formaldehyde 1:2,500, mercuric chloride 1:1000, copper sulfate 1:350, and a mixed preservative consisting of formaldehyde 1:10,000, mercuric chloride 1:8,000 and thymol 1:4,000.

A large sample of milk was divided into 17 sub-samples each consisting of about 500 cc., which were then set aside at the desired temperature after the addition of the preservative. The containers for the samples were glass bottles. These were filled about nine-tenths full and well stoppered. One sub-sample was analyzed at once for fat, lactose, and the various protein constituents. Bacteriological counts were made at the same time. One set of the sub-samples was analyzed similarly at the end of three weeks and another at the end of fourteen weeks. The data from this experiment are given in Table 2.

The principal result of this experiment was the failure to confirm the results secured in the preliminary experiments showing the efficiency of formaldehyde as a preservative of the protein constituents of milk when used in the proportion of one part of 2,500 of milk. Appreciable proteolysis occurred in all the samples containing formaldehyde. The samples containing the mixed preservative were also very poorly preserved. Copper sulfate in the proportion of one part to 350 of milk apparently preserved the milk well at 2°C. for a period of three weeks, but considerable decomposition had taken place at the end of fourteen weeks.

In general the experiment shows clearly the importance of the temperature at which the samples are kept. In most of the samples there was a greater proteolysis in the samples kept at 14°C. in comparison with the corresponding samples kept at 2°C.

Another interesting result of this experiment was the apparently greater germicidal effect of mercuric chloride in comparison with the other preservatives.

TABLE 2.—INFLUENCE OF TEMPERATURE ON PRESERVATION

Preservative	Conc. of preservative	Temperature of preservation	Time of analysis	Casein N.	Heat-coag. Prot. N.	Residual Prot. N.	Total Alb. and Glob. N.	Resid. Non-p. N.	Total N.	Fat	Lactose	No. of Bac. per cc.	No. of moulds per cc.
		°C	*days*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
.....	.....	....	at once	0.348	0.074	0.045	0.119	0.035	0.495	3.59	4.82	40,000	000
HCHO	1:2,500	2	21	.310	.086	.060	.146	.035	.495	3.57	4.80	25,400	70,000
HCHO	1:2,500	2	98	.309	.058	.088	.146	.039	.495	3.77	.....	850	8,920
HCHO	1:2,500	14	21	.321	.070	.066	.136	.037	.495	3.54	4.80	2,500	62,400
HCHO	1:2,500	14	98	.301	.045	.085	.129	.056	.495	3.48	.....	600	3,700
$HgCl_2$	1:1,000	2	21	.325	.111	.027	.138	.036	.495	3.61	4.81	000	000
$HgCl_2$	1:1,000	2	98	.309	.096	.052	.148	.039	.495	3.63	.....	160	80
$HgCl_2$	1:1,000	14	21	.298	.111	.042	.153	.036	.495	3.51	4.80	000	000
$HgCI_2$	1:1,000	14	98	.263	.091	.088	.179	.047	.495	3.61	.....	400	12,000
$CuSO_4$	1:350	2	21	.351	.083	.031	.114	.035	.495	3.44	4.80	20,700	400
$CuSO_4$	1:350	2	98	.304	.081	.063	.144	.041	.495	3.63	.....	12,800	1,400
$CuSO_4$	1:350	14	21	.318	.081	.060	.141	.035	.495	3.49	4.78	637,000	000
$CuSO_4$	1:350	14	98	Spoiled									
Complex	.....	2	21	.318	.081	.060	.141	.035	.495	3.59	4.77	123,000	20
Complex	.....	2	98	.279	.085	.093	.178	.042	.495	3.61	.....	300,000	20
Complex	.....	14	21	.282	.091	.085	.176	.041	.495	3.15	4.77	2,277,000	35
Complex	.....	14	98	.224	.067	.133	.200	.061	.495	3.51	.....	500	600

INFLUENCE OF DEVELOPMENT OF BACTERIA BEFORE ADDITION OF PRESERVATIVE

The object of this experiment was to study the influence of the development of a large number of bacteria before the addition of the preservative to the milk. This was accomplished by allowing the milk to stand for a period of time at 14°C. before adding the preservative. Two preservatives were used in this study, namely, formaldehyde in the proportion of one part to 2,500 of milk and the mixed preservative used in the previous experiment.

The technic of the experiment was as follows: A large sample of perfectly fresh milk was cooled at once to 14°C. and two sub-samples taken, one for immediate analysis and the other set aside at 2°C. after the addition of formaldehyde. The remainder of the milk was allowed to stand at 14°C. for 24 hours when three more sub-samples were taken, one for immediate analysis, and the other two set aside at 2°C. and 14°C., respectively, after the addition of formaldehyde. The remainder of the milk was then allowed to stand for 24 hours more when five sub-samples were taken, one for immediate analysis, two more treated as at the end of the first 24 hours and the remaining two in a similar way except that the mixed preservative was added to them. The remainder of the milk was then allowed to stand for 24 hours more, when five sub-samples were taken and treated as those withdrawn at the end of the second 24 hours.

All the sub-samples were analysed at the end of 26 days standing at the temperatures specified, with the exception of those indicated for immediate analysis.

Besides the analyses for fat, lactose and protein constituents, counts were made in all the samples for the number of bacteria and moulds. The results are given in Table 3.

The general result of this experiment was to show that an appreciably depreciatory influence is exerted upon the preservation of the milk constituents by allowing the milk to stand for more than 24 hours at 14°C. before adding the preservative. Even when the milk was subsequently kept at 2°C. marked proteolysis of the casein characterized the samples which stood longer than 24 hours before adding the preservative. When the samples were kept at 14°C. thruout the experimental period of 26 days proteolysis was marked in all cases, showing that the temperature at which they were preserved was a greater factor than the bacterial development which took place before the preservative was added. The lactose, also, was decomposed appreciably in these cases.

TABLE 3.—INFLUENCE OF DEVELOPMENT OF BACTERIA BEFORE PRESERVATION

Preservative	Temperature	Casein N.	Heat-coag. N.	Residual Prot. N	Total Alb.and Glob.N.	Residual Non-prot. N.	Total N.	Fat	Lactose	Bacteria per cc.	Moulds per cc.	When analyzed	Time standing at 14°C. before pres. added.
	°C	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*				*hours*
None	14	0.367	0.078	0.041	0.119	0.041	0.528	3.68	5.15	210	0	at once	....
None	14	.364	.085	.036	.121	.041	.525	3.56	5.13	10,000	0	after 24 hrs.	....
None	14	.362	.086	.041	.127	.041	.527	3.65	5.11	550,000	0	" 48 "	....
None	14	.353	.081	.049	.130	.041	.525	3.63	4.95	39,000,000	0	" 72 "	....
HCHO 1:2500	2	.364	.071	.041	.112	.041	.528	3.54	.....	1,000	0	after 26 days	1
HCHO 1:2500	2	.364	.075	.042	.117	.041	.525	3.70	5.15	800,000	0	" 27 "	24
HCHO 1:2500	2	.353	.078	.056	.134	.041	.527	3.55	4.93	1,036,600	0	" 26 "	48
HCHO 1:2500	2	.338	.077	.071	.148	.041	.525	3.52	4.89	24,000	0	" 26 "	48
Complex	2	.361	.083	.053	.136	.034	.527	3.65	5.02	1,380,000	0	" 26 "	48
Complex	2	.336	.093	.063	.156	.039	.525	3.65	4.94	1,304,000	0	" 26 "	72
HCHO 1:2500	14	.277	.056	.069	.125	.125	.525	.....	4.46	41,730	1,120	" 27 "	24
HCHO 1:2500	14	.328	.078	.073	.151	.060	.527	3.35	4.90	50,400	0	" 26 "	48
HCHO 1:2500	14	.321	.081	.066	.147	.067	.575	3.75	4.34	908,000	4,000	" 26 "	72
Complex	14	.299	.049	.088	.137	.089	.527	3.18	4.51	78,230	0	" 26 "	48
Complex	14	.332	.089	.061	.150	.045	.525	3.90	.....	625,000	0	" 26 "	72

The gradual hydrolysis of the casein when fresh milk is allowed to stand at 14°C. is strikingly shown by the data for the first four samples in Table 3. There seems to have been some relation between the results of the analyses in these cases and the development of bacteria. The decomposition of the lactose apparently did not begin until after the milk had stood for 48 hours.

In general, it may be stated that the experiment does not reveal any consistent relation between the number of bacteria in the milk at the end of 26 days and the extent of the proteolysis which had taken place during this time.

INFLUENCE OF AIR ON PRESERVATION

The object of this experiment was to study the influence of air upon the preservation of the milk constituents, especially thru its relation to the growth of mould. The preservatives used were formaldehyde in the proportion of one part to 2,500 of milk and the mixed preservative used in the two preceding experiments. The plan of the experiment, together with the results secured are shown in Table 4.

The results of this experiment show that considerable air in contact with the milk has a marked influence upon the preservation of the milk constituents, aparently very appreciably increasing the proteolysis of all the protein constituents. The results show no connection, however, between this effect and the number of mould organisms which developed in the individual samples of milk. The figures show that the mould growth was relatively large in all the samples containing formaldehyde alone altho the amount of air in contact with the milk varied from none at all to a large quantity. Furthermore, at the end of 56 days the composition of the two samples preserved with the mixed preservative at 2°C. was almost identical with that of the corresponding samples preserved with formaldehyde alone altho the number of mould organisms in the samples containing the mixed preservatives was insignificant compared with the number in the samples containing formaldehyde alone.

The best preservation was obtained when the sample bottles were nine-tenths full. The cause of the detrimental effect of more or less air than this is not apparent.

This experiment gives an additional example of the fact that formaldehyde in the proportions of one part to 2,500 of milk is

TABLE 4.—INFLUENCE OF AIR ON PRESERVATION

Preservative	Conc. of Pres.	Temp. of Pres.	Quantity of milk in bottle	Time of analysis	Casein N.	Heat-coag. Protein N.	Residual Prot. N.	Total Alb. and Glob. N.	Residual Non-Prot. N.	Total N.	Fat	Lactose	Bacteria per cc	Moulds per cc.
		°C	*per cent*	*days*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*		
None	.....	....	.....	at once	0.348	0.074	0.047	0.121	0.035	0.494	3.59	4.82	40,000	0
HCHO....	1:2,500	2	25	56	.303	.069	.081	.150	.053	.494	.....	4.74	288,000	821,600
HCHO....	1:2,500	2	90	56	.316	.067	.068	.135	.038	.494	3.39	4.94	122,000	749,000
HCHO....	1:2,500	14	50	56	.266	.054	.097	.151	.078	.494	3.48	4.86	77,000	533,000
HCHO....	1:2,500	14	100	56	.293	.064	.091	.155	.049	.494	3.68	4.74	3,902,000	710,000
Complex	.....	2	25	56	.305	.067	.081	.148	.038	.494	3.48	4.86	902,000	1,400
Complex	.....	2	90	56	.318	.067	.078	.145	.038	.495	3.36	5.01	802,000	11,850
Complex	.....	14	50	56	.233	.056	.127	.183	.081	.495	3.38	5.02	965,000	1,500
Complex	.....	14	100	56	.274	.069	.100	.169	.049	.495	3.72	4.94	1,593,800	16,900

not a perfect preservative of the milk constituents even when the samples are kept at 2°C.

RELATIVE IMPORTANCE OF ENZYMES AND BACTERIA IN PRESERVATION OF MILK

The results secured in the experiments so far reported seem to justify the general conclusion that formaldehyde, under certain conditions, is probably as efficient a preservative of the various constituents of milk as any which is available at the present time. The best conditions which have been brought out so far are: (1) a concentration of formaldehyde of 0.04 per cent, or one part of formaldehyde to 2,500 of milk, (2) addition of the formaldehyde as soon as possible after drawing the milk, (3) leaving just sufficient air in contact with the sample to allow mixing, i. e., filling the bottle about nine-tenths full and (4) keeping the sample at 2°C.

The variable results obtained even under the foregoing conditions shows very clearly, however, that the chief factor causing the decomposition of milk in these cases was still not under perfect control. It seems very probable that this factor is the natural proteoclastic enzyme, the galactase of the milk. A number of investigators, whose results have been reviewed in the preceding pages, have shown that formaldehyde merely retards the activity of this enzyme. Therefore it seemed advisable to give this question some study, particularly with reference to the relative importance of bacteria and enzymes in the preservation of the various milk constituents.

The night's milking from two individual cows was placed in separate covered cans and immediately brought to the cooler in which a temperature of 4-5°C. was maintained. The next morning five separate sub-samples were taken from each can and placed in 32-ounce glass-stoppered bottles. Each bottle was filled nine-tenths full. Each set of five samples was treated as follows: Sample 1 received no preservative and was analysed at once; Samples 2 and 3 received formaldehyde in the proportion of one part to 2,500 of milk, Sample 2 being analysed at the end of two weeks and Sample 3 at the end of six weeks; Samples 4 and 5 received 1.5 per cent chloroform, Sample 4 being analysed at the end of two weeks and Sample 5 at the end of six weeks. All the samples were kept at approximately 14°C. The fat in this and the subsequent experiments was determined by the Babcock centrifugal method.

It was expected that the samples containing chloroform would show considerably more proteolysis than the samples containing for-

maldehyde if the inconsistencies of the results previously obtained were due to the activity of galactase. This expectation was based on the results secured by Harding and Van Slyke[25] who found that 1.5 per cent chloroform acted as a strong germicide but did not retard the activity of the proteoclastic enzyme of milk.

It seemed advisable as a preliminary test to determine whether 1.5 per cent chloroform in the milk would have any effect upon the proportion of the protein constituents of milk as determined by the methods of analysis which had been adopted for these studies. For this purpose a sample of milk was divided into two parts; formaldehyde was added to one part in the proportion of one part to 2,500 of milk, and 1.5 per cent chloroform was added to the other part. The two samples were then analyzed at once for the various protein constituents. The results of the test are given in Table 5. The data show that no effect is produced upon the usual distribution of nitrogen in milk due to the presence of this amount of chloroform.

TABLE 5.—COMPARISON PROTEIN DISTRIBUTION IN MILK PRESENCE OF HCHO 1:2,500 AND $CHCl_3$ 1.5 PER CENT

Constituent	HCHO 1:2,500	$CHCl_3$ 1.5 per cent.
Casein N. ..... per cent	0.406	0.400
Heat-coagulable protein N. ..... per cent	.060	.065
Residual protein N. ..... per cent	.050	.056
Total albumin and globulin N. ..... per cent	.118	.121
Residual non-protein N. ..... per cent	.030	.030
Total N. ..... per cent	.553	.553

In the experiment proper the results of the analyses of the samples of milk from the two cows were characterized by such a uniformity that it seemed permissible to present the data in the form of an average. The data from this experiment are shown in Table 6.

A study of the data in this table leads to the conclusion that the most important factor to be controlled in bringing about a satisfactory preservation of the protein constituents of milk is the action of the proteoclastic enzyme galactase. Under the conditions of this experiment formaldehyde in the proportion of 1 to 2,500 of milk greatly retarded this action but did not prevent it entirely. Satisfactory preservation was secured for two weeks with formaldehyde but the samples did not show an analysis identical with the fresh milk after standing for a period of six weeks.

TABLE 6.—COMPARATIVE PRESERVATION OF MILK IN PRESENCE OF HCHO, 1:2,500 AND $CHCl_3$, 1.5% RESPECTIVELY

Constituent	Analysis of fresh milk	Milk preserved with 1.5% $CHCl_3$		Milk preserved with HCHO, 1:2,500	
		After 2 weeks	After 6 weeks	After 2 weeks	After 6 weeks
Casein N.—per cent	0.368	0.318	0.260	0.364	0.354
Heat-coag. protein N.—per cent	.046	.067	.073	.050	.038
Residual protein N.—per cent	.061	.073	.108	.055	.072
Total albumin and globulin N.—per cent	.107	.140	.181	.105	.102
Residual non-protein N.—per cent	.031	.040	.043	.034	.038
Total N.—per cent	.505	.505	.505	.505	.505
Fat—per cent	3.65	3.65	3.65	3.49	3.49
Lactose—per cent	4.94	4.87	4.84	4.87	4.78
Peroxidase—per cent	+++	++++	++++	++++	+
Catalase—per cent	47	68	58	10	15
Acid bacteria per cc.	85,000	0	0	35,000	15
Peptonizing bacterial per cc.	52,000	0	0	0	15
Alkaline and inert bacteria per cc.	9,885,000	0	0	40,000	70
Total bacteria per cc.	10,022,000	575	10	75,000	100
Moulds per cc.	1,075,000	0	0	900	7,350

INFLUENCE OF INCUBATION WITH PRESERVATIVE ON DESTRUCTION OF BACTERIA AND ENZYMES

The experiments so far conducted show very clearly that the two most important conditions to be secured for the efficient preservation of the various constituents of milk are that the bacterial of the milk be destroyed and especially that the action of the proteoclastic enzymes be inhibited.

As has already been stated, the best preservative found in the studies made thus far is formaldehyde in the proportion of one part to 2,500 of milk when used under certain conditions, which have been mentioned. One of the most important of these conditions, namely, keeping the milk at a low temperature, is frequently not feasible under practical laboratory routine. The temperature most feasible for most laboratories to maintain is that of the well cooled ice box, which is 8°-10°C. All the remaining experiments were accordingly planned with this temperature as a basis. The experiments so far reported show, however, that efficient preservation can not be expected at a temperature of 10°C. without introducing some controlling factors other than those already mentioned.

Formaldehyde is recognized as a strong germicidal agent and the data presented in Table 7 show that this effect is considerably

TABLE 7.—INCREASED GERMICIDAL EFFECT OF HCHO AT INCUBATION TEMPERATURE

Character of test	Bacteria per cc.		
	At start	After 6 hours	After 48 hours
HCHO, 1:2,500, placed in cooler at 10°C.	10,000	1,700	100
HCHO, 1:2,500, placed in incubator at 37°C	10,000	20	5

augmented by allowing the formaldehyde to act at the temperature most favorable for the growth of most bacteria, namely, 37°C. This result suggested the possibility that a similar procedure might also decrease the activity of the proteoclastic enzymes. It has already been pointed out that Jensen[10] found the proteoclastic enzymes of milk to be no more active at 35°C. than at room temperature in the presence of 1.0 per cent formalin (formaldehyde 1:250). He attributed this result to the fact that the greater activity of the enzymes at 35°C. is counterbalanced by the greater antiseptic power of the formaldehyde at the higher temperature. In Jensen's experiments the samples were kept continuously at the temperatures studied. It seemed therefore worth while to investigate whether a short incubation in the presence of formaldehyde would result in a more favorable destruction of the enzymes of the milk, particularly those which accelerate the hydrolysis of the proteins.

EXPERIMENT 1.—A sample of fresh milk was divided into three portions, and each portion placed in a 32-ounce glass-stoppered bottle filled nine-tenths full. One of the samples was analyzed immediately. Formaldehyde 1:2,500 was added to each of the remaining samples and one was placed in the cooler at 8°-10°C. and the other in the incubator at 37°C. for 48 hours and then in the cooler. Both of these samples were analyzed at the end of six weeks. The results of the experiment are given in Table 8.

TABLE 8.—INFLUENCE OF INCUBATION WITH FORMALDEHYDE ON SUBSEQUENT PROTEOLYSIS AND OTHER CHANGES IN MILK

Constituent	Analysis of fresh milk	HCHO1:2,500. After 6 weeks at 10°C.	HCHO1:2,500. 48 hours at 37°C. After 6 weeks at 10°C.
Casein N.—per cent	0.370	0.357	0.361
Heat-coag. protein N.—per cent	.050	.036	.038
Residual protein N.—per cent	.067	.075	.072
Total albumin and globulin N.—per cent	.117	.111	.110
Residual non-protein N.—per cent	.030	.050	.034
Total nitrogen—per cent	.517	.517	.517
Fat—per cent	3.22	3.22	3.22
Lactose—per cent	4.98	4.76	4.96
Oxidase	None	None	None
Perodidase	++	+	None
Catalase—per cent	57	22	25
Acid bacteria per cc.	20,000	20	0
Peptonizing bacteria per cc.	30,000	20	0
Alkaline and inert bacteria per cc.	850,000	60	0
Total bacteria per cc.	900,000	100	0
Moulds per cc.	450,000	13,000	0

The data show that the effect of incubation was to increase greatly the germicidal power of the formaldehyde. There was, however, very little increased inhibition of the activity of the proteoclastic enzymes as the result of the incubation.

Altho this experiment failed to give satisfactory evidence that incubation at 37°C. gives an effective enzyme antisepsis the question seemed worthy of more extended study, particularly with reference to the effect of greater concentrations of formaldehyde than the one used in this experiment.

EXPERIMENT 2.—A portion of a sample of milk was set aside for immediate analysis for percentage of total soluble protein nitrogen and the remainder of the milk sub-divided into a number of samples to which various preservatives were added. These subsamples were kept at 37°C. for two weeks, when the percentage of soluble nitrogen was again determined. The results of the experiment are shown in Table 9.

The data from this experiment show very clearly the relative antiseptic power of the various preservatives toward proteolysis. The relative efficiency of the different concentrations of formaldehyde is also strikingly shown.

The marked proteolysis of the samples containing toluene and benzene in comparison with the samples containing the same concen-

tration of these preservatives plus formaldehyde demonstrates clearly that the proteolysis with which we are dealing in the preservation of milk is of enzyme origin. In addition these results indicate that formaldehyde is probably the most powerful antiseptic toward this enzyme which is available at the present time. According to E. Fisher[26] toluene has scarcely any destructive action toward enzymes, while it prevents the growth of protoplasmic structures.

TABLE 9.—INFLUENCE OF INCUBATION WITH VARIOUS PRESERVATIONS UPON PROTEOLYSIS

Preservative	Soluble protein N. at start.	Soluble protein N. after 2 weeks
HCHO 1:2,500	0.151	Spoiled
HCHO 1:1,250	0.151	0.149
HCHO 1:625	0.151	0.144
$HgCl_2$ 1:1,000	0.151	0.204
Benzene 1:20	0.151	0.270
Toluene 1:20	0.151	0.274
HCHO 1:2,500, Benzene. 1:20	0.151	0.141
HCHO 1:2,500, Toluene. 1:20	0.151	0.141

Note: The results of the analyses of the samples containing benzol and toluene were corrected for volume of these reagents added.

Of the several concentrations of formaldehyde used in this experiment one part to 1250 parts of milk is shown to be the best. The curdling of the sample containing one part of formaldehyde to 2,500 parts of milk shows that this concentration was not sufficiently great to prevent the development of lactic acid bacteria at the incubation temperature, while the concentration of formaldehyde of 1:625 evidently caused the precipitation of some of the albumin as casein, as was shown by Patrick[18] and his co-workers to take place with this concentration of formaldehyde.

EXPERIMENT 3.—The object of this experiment was to show the effect of a 48-hour incubation with formaldehyde 1:1250 upon the preservation of the various milk constituents, the milk to be kept subsequently at about 10°C. For comparative purposes other samples of the same milk were (1) kept at the incubation temperature, (2) kept at about 10°C. without previous incubation, (3) kept at room temperature without previous incubation, and (4) incubated for 48 hours and kept at room temperature.

A large sample of milk was subdivided as in the other experiments of this characater, and one portion analyzed immediately.

The other sub-samples, to which preservative was added, were placed in 32-ounce glass-stoppered bottles, filled nine-tenths full. The duration of the preservation period was thirty days. The data from this experiment are given in Table 10.

TABLE 10.—INFLUENCE OF INCUBATION TEMPERATURE WITH HCHO 1:1,250 ON PRESERVATION OF MILK CONSTITUENTS

Constituent	Analysis of fresh milk	Analyses after six weeks				
		Kept at 37°C.	37°C. for 48 hours then 10°C.	Kept at 10°C.	Kept at 20°C.	37°C for 48 hours then at 20°C.
Casein N.—per cent	0.446	0.446	0.457	0.444	0.431	0.450
Heat-coag. protein N.—per cent	.053	.024	.034	.036	.030	.028
Residual protein N.—per cent	.049	.072	.056	.064	.069	.060
Total albumin and globulin N.—per cent	.112	.096	.090	.100	.099	.088
Residual non-protein N.—per cent	.028	.033	.028	.030	.035	.027
Total N.—per cent	.577	.577	.577	.577	.577	.577
Fat—per cent	4.10	3.90	3.90	3.90	3.90	4.00
Lactose—per cent	5.02	5.02	5.02	5.02	5.02	5.00
Oxidase	None	None	None	None	None	None
Peroxidase	+++	"	"	+++	+	"
Catalase—per cent	30	20	20	20	20	20
Bacteria per cc	3,000	50	120	200	500	100

The results obtained in this experiment were somewhat surprising in that the sample preserved at 10°C. without previous incubation proved to be the best preserved at the end of thirty days. The loss of casein and soluble proteins in this sample was, in fact, negligible. The data indicate that for efficient preservation of the protein constituents of milk using formaldehyde as preservative, an amount considerably in excess of that usually recommended must be used.

The experiment shows clearly the effect of formaldehyde upon the relative distribution of heat-coagulable and residual protein in the milk. The data therefore serve to emphasize the fallacy of drawing conclusions regarding the albumin content of milk based upon its estimation by coagulation with heat.

THE MINIMUM QUANTITY OF FORMALDEHYDE FOR PRESERVATION

In order to determine more closely the minimum quantity of formaldehyde which will serve as an efficient preservative of the milk constituents, especially the milk proteins, a further experiment was planned.

A large sample of fresh milk was divided into 15 sub-samples which were placed in 32-ounce glass-stoppered bottles filled nine-tenths full. These sub-samples were then divided into five groups of three each, to which were added formaldehyde in the proportions of 1:1,400, 1:1,600, 1:1,800, 1:2,000 and 1:2,200, respectively. One of the three samples of each group was analysed immediately. Another sample of each group was placed in the refrigerator at about 10°C. The third sample of each group was placed in the incubator at 37°C. The samples in the refrigerator were analyzed at the end of five days.

TABLE 11.—INFLUENCE OF DIFFERENT CONCENTRATIONS OF HCHO ON PRESERVATION OF MILK

Constituent	When analyzed	Proportion of HCHO in sample				
		1:1,400	1:1,600	1:1,800	1:2,000	1:2,200
Casein N.	at once	0.379	0.379	0.379	0.379	0.379
Casein N.	after 5 days at 37°C.	.392	.386	.377	.373	.370
Casein N.	after 5 days at 10°C.	.378	.373	.365	.372	.359
Total albumin and globulin N.	at once	.105	.105	.105	.105	.105
Total albumin and globusin N.	after 5 days at 37°C.	.097	.102	.108	.110	.113
Total albumin and globusin N.	after 5 days at 10°C	.108	.110	.119	.113	.121
Residual non-protein N.	at once	.033	.033	.033	.033	.033
Residual non-protein N.	after 5 days at 37°C	.036	.034	.036	.038	.039
Residual non-protein N.	after 5 days at 10°C	.031	.034	.034	.033	.033
Total N.	at once	.517	.517	.517	.517	.517
Total N.	after 5 days at 37°C	.524	.522	.518	.518	.522
Total N.	after 5 days at 10°C	.518	.517	.517	.517	.511

The data from this experiment are presented in Table 11. They indicate clearly that the minimum quantity of formaldehyde necessary to preserve efficiently the protein constituents of milk, using

ordinary cooling facilities, is approximately one part to 1,700 of milk. Amounts in excess of this up to one part to 1,400 of milk appear to be permissible also. When incubation is used to facilitate the destruction of bacteria and enzymes it is apparently impracticable to use formaldehyde in excess of one part to 1,800 of milk without affecting the determination of casein and soluble proteins.

THE PRESERVATION OF A COMPOSITE SAMPLE

Studies involving the chemical analysis of milk are not always made on samples representing merely one milking or one day's yield of milk but are frequently made on samples representing several days' yield and more often on the milk of an entire week. In such cases a composite sample is made of the days to be represented and at the end of the sample period a sub-sample is taken from the composite which represents the average composition of the milk during that period. A study of the preservation of milk for chemical analysis would therefore be incomplete without extending the results to a study of their applicability to the preservation of a composite sample.

It was planned to make all the conditions of this experiment as practical as possible. None of the samples were subjected to incubation, as previous experience had not given results of sufficient value to warrant this step. Instead, the samples were kept in the refrigerator at about 10°C. The preservative used was formaldehyde in the proportion of one part to 1,500 of milk, added, as in all other experiments, in the form of formalin.

The night and morning milkings of a selected cow were brought to the refrigerator immediately after milking. In the morning the two milkings were combined and treated as follows:

1. The milk was mixed thoroly by pouring from can to can.
2. A 32-ounce glass-stoppered bottle was filled nine-tenths full of the thoroly mixed milk. The bottle was labeled Sample I, and was set aside for immediate analysis.
3. The remaining milk was carefully measured and formaldehyde added in the proportion of one part to 1,500 of milk.
4. A 32-ounce glass-stoppered bottle was filled nine-tenths full of the formaldehyde treated milk. The bottle was labeled Sample 1, and placed in the refrigerator.
5. Ten per cent of the measured, formaldehyde-treated milk of Step 3 was placed in a five-liter bottle for the composite sample. This bottle was kept at about 10°C.

At the end of three days another night and morning milking from the same cow was treated according to the steps given above. The sample of fresh milk for immediate analysis was labeled Sample II. The formaldehyde-treated milk in Step 4 was labeled Sample 2. In Step 5 the ten per cent of the measured milk which was withdrawn was added to the five-liter bottle containing the similar sample prepared three days previously.

At the end of three more days the same procedure was carried out. The sample of fresh milk was labeled Sample III. The sample of formaldehyde-treated milk taken in Step 4 was labeled Sample 3. Ten per cent of the measured milk was added to the five-liter bottle set aside for that purpose.

After the third portion of the composite was added to the five-liter bottle the contents were thoroly mixed and two sub-samples taken in 32-ounce bottles, as in the other samples, and labeled Sample IV and Sample VI, respectively. Sample IV was set aside for immediate analysis, and Sample VI analyzed at the end of three weeks in the refrigerator. At the time of analysis of Sample VI the sub-samples labeled 1, 2 and 3 were united and a sub-sample taken from the mixture. This was labeled Sample V and was analysed with Sample VI.

The data from this experiment are shown in Table 12. They show that the method used in this experiment for making a com-

TABLE 12.—PRESERVATION OF A COMPOSITE SAMPLE FOR 3 WEEKS WITH HCHO 1:1,500

Sample:	Casein N.	Albumin and globu-tein N.	Residual non-pro-lin N.	Total N.	Fat	Lactose
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
I*	0.439	0.102	0.028	0.572	5.05	5.47
II**	.445	.111	.031	.586	5.12	5.47
III†	.417	.114	.028	.560	4.90	5.47
IV††	.434	.113	.028	.569	5.05	5.47
V‡	.431	.110	.028	.566	4.95	5.40
VI‡‡	.431	.107	.027	.566	4.95	5.40

*First day.
**Third day.
†Sixth day.
††Composite of I, II, & III analyzed at once.
‡Composite of duplicates of I, II, & III made and analyzed at end of 3 weeks.
‡‡Duplicate of Sample IV analyzed after 3 weeks.

posite sample and preserving it for future study gave a satisfactory preservation of the milk constituents. The two composites, namely, Samples V and VI, were practically identical with one another and also with Sample IV, in composition.

SUMMARY AND CONCLUSIONS

In this bulletin a detailed study of the preservation of milk for chemical analysis is reported, particularly with reference to the preservation of the protein constituents.

The experiments which were carried out were designed to show the influence of the following factors upon the preservation of milk: (1) The kind of preservative; (2) the temperature of preservation; (3) the development of bacteria before adding the preservative; (4) the amount of air in contact with the milk; (5) the relative importance of bacteria and enzymes in causing decomposition; and (6) the minimum quantity of the best preservative to use.

The preservatives selected for comparison were formaldehyde, mercuric chloride, potassium dichromate, copper sulfate, thymol, and toluene. Formaldehyde is shown to be the most efficient of these reagents in the comparisons which were made.

It is shown that milk is preserved best at a temperature near the freezing point but an effort was made to establish the proper conditions for an equally efficient preservation at a temperature of approximately 10°C., as being more practicable for the average laboratory to maintain.

It was found inadvisable to allow milk to stand for more than twenty-four hours at 14-15°C. before adding the preservative. The data in regard to this factor suggest that milk which is to be preserved for future chemical analysis should be cooled at once to the temperature at which it is to be preserved and the preservative added immediately.

The amount of air in contact with the sample of milk is shown to be an important factor in efficient preservation, more than just enough air to permit subsequent mixing of the sample being detrimental to the preservation of the milk. Filling the sample bottles nine-tenths full was found to be a safe practice.

The destruction of enzymes, particularly the natural proteoclastic enzymes of the milk, is shown to be the most important condition to be secured for the efficient preservation of milk, since most preservatives are much stronger germicides than inhibitors of enzyme action.

A study of the effect of holding the milk for a short time at 37°C. in the presence of formaldehyde, on the destruction of the natural proteoclastic enzyme of milk did not result in sufficiently greater efficiency to warrant the recommendation of the procedure in actual practice.

Proportions of formaldehyde not less than one part to 1,700 of milk and not greater than one part to 1,250 are shown to be the most efficient quantities of this preservative to add to milk to bring about a satisfactory preservation of the various constituents for three to four weeks.

It is shown, however, that these quantities of formaldehyde do not permit the use of heat-coagulation for determining the "albumin" of milk. Some discussion is given to the use of this method of analysis under any conditions, and its limitations pointed out.

A study of the preservation of a composite sample using formaldehyde 1:1,500 as preservative led to satisfactory results.

A study of the entire data in this bulletin suggest that the following procedure can be safely recommended as a satisfactory one for the preservation of all the constituents of milk for a period of several weeks.

METHOD RECOMMENDED FOR PRESERVATION OF MILK

Mix the sample thoroly as soon as drawn; measure carefully one liter and add between 1.5 and 2.0 cc. of formalin (containing approximately 40 per cent formaldehyde); place a suitable portion in a bottle, preferably a glass-stoppered one, filling the bottle about nine-tenths full; cool at once to 8-10°C. or lower and maintain at that temperature until ready for analysis.

ACKNOWLEDGMENT

This investigation was begun at the suggestion of Dr. C. H. Eckles whose advice and criticism are gratefully acknowledged. The data were taken in cooperation with the Dairy Division, U. S. Department of Agriculture during the years 1911 to 1913. The assistance of Mr. Roy Lee Primm is acknowledged in connection with part of the analytical work.

BIBLIOGRAPHY

[1]U. S. Dept. of Agr., Bureau of Chemistry Bul. 107 (Revised), 1908; p. 117.

[2]A very complete review of the subject of milk preservation for chemical analysis up to the year 1908 is given by Windish, Karl. Die Conservierung der Milchproben für analytische Zwecke. Milchwirtschaft. Zentralblatt, 1908. 4: 97-108.

[3]Price, T. M. Influence of formaldehyde on the digestive enzymes. 20th Ann. Rep. U. S. Dept. of Agr., Bureau of Animal Industry, 1903, pp. 114-121.

[4]Rivas, D. Study of formaldehyde in milk. Univ. of Pa. Medical Bul., 1904. 17: 175-180.

[5]Seligman E. Uber den Einfluss einiger Aldehyde, besonders Formalins, auf die Oxydationsfermente der Milch und des Gummi arabikums. Zeit. f. Hyg., 1905. 50: 97-122.

[6]Schaps, Leo. Sur Frage der Conservierung der Milch durch Formaldehyde, speciell zum Zwecke der Säuglingsernährung. Zeit. f. Hyg., 1905. 50:246-261.

[7]Sommerfeld, Paul. Uber Formalinmilch und das Verhalten von Formalin gegenüber einigen Bakterienarten. Zeit. f. Hyg. 1905. 50: 153-164.

[8]Babcock, S. M., Russell, H. L., and Vivian, Alfred. Properties of galactase; A digestive ferment of milk. 15th Ann. Rep. Wis. Agr. Exp. Sta., 1898. pp. 77-86.

[9]Freudenreich, Ed. Uber das in der Milch vorhandene unorganisierte Ferment die sogenannte Galactase. Landw. Jahrb. Schweiz, 1900. 14: 49-55.

[10]Jensen, Orla. Studien über die Enzyme in Käse. Landw. Jahrb. Zchweiz, 1900. 14: 197-233.

[11]Van Slyke, L. L., Harding, H. A., and Hart, E. B. A study of enzymes in cheese. N. Y. Agr. Exp. Sta. Bul. 203: 165-193, 1901.

[12]Tice, W. G. and Sherman, H. C. Proteolysis in cows' milk preserved by means of formaldehyde. J. Am. Chem. Soc. 1906. 28: 189-194.

[13](a) Bliss, C. L., and Novy, F. G. Action of formaldehyde on enzymes and on certain proteids. J. Exp. Med. 1899. 4: 47-48.

(b) Lowenstein, E. Die Wirkung des Formalins auf die Milch und das Lab. Zeit. f. Hyg. 1904. 48: 239.

(c) Price, T. M. (see reference 3).

(d) Bandini, P. Wirksamkeit des Formalins und das Wassertsoffsuperoxyds in der Milch. Cent. f. Bact. 1906. 41: Abt 1, pp. 271, 379, 474.

[14]Cochran, C. B. Milk preservatives. Ann. Rep. Pa. Dept. of Agr. 1899. pp. 277-289.

[15]Blum, F. Uber eine Klasse von Verbindungen der Eiweisskörper. Z. physiol. Chem. 1896-7. 22: 127-136.

[16]Bach, A. Action of formaldehyde on albumin. Mon. Sci. 1897. (iv)

11: 157-159. in Jour. Chem. Soc. Abstracts, 1897. 74: pt. 1, p. 287.

[27]Cavanaugh, G. W. Report on dairy products. 18th Ann. Rep. A. O. A. C. U. S. Dept. of Agr., Bureau of Chem. Bul. 73: p. 37, 1903.

[28]Patrick, G. E. Report on dairy products. 21st Ann. Rep. A. O. A. C. U. S. Dept. of Agr., Bureau of Chem. Bul. 90; pp. 78-83, 1905; 22nd Ann. Rep. A. O. A. C. U. S. Dept. of Agr. Bureau of Chem. Bul. 99: pp. 94-99. 1906.

[29]Tillmans, J., Splittgerber, A. and Riffart, H. Uber die Konservierung von Milchproben zu Untersuchszwecken, Z. Nahr. Genussm. 1914. 27: 893-901.

[30]Gerber, C. The action of mercury salts on the coagulation of milk by proteolytic enzymes. I. Mercuric chloride and vegetable rennins with boiled milk. Comp. Rend. Soc. Biol. 1910. 68: 631-3: 765-8. in Chem. Abs. 1910. 4: 2328.

[31]Kühn, M. The keeping of milk for analysis. Mölk. Zeitung, 1894. 18: 354. in J. Chem. Soc. Abstracts. 1895. 68: pt. II, p. 189.

[32]Richmond, Henry Droop. Dairy Chemistry, 2nd edition, revised. 1914, p. 190. Charles Griffin and Co., Limited, London.

[33]Five grams of pure tannic acid are dissolved in 240 cc. of 50 per cent alcohol and 10 cc. of 25 per cent acetic acid solution added. The solution is filtered, if necessary.

[34]Rogers, L. A. Berg, W. N., and Davis, B. J. The temperature of pasteurization for butter making. U. S. Dept. of Agr. Bureau of Animal Industry Circular 189, pp. 312-315, 1912.

[35]Harding, H. A., and Van Slyke, L.L. Chloroform as an aid in the study of milk enzymes. N. Y. Agr. Exp. Sta. Tech. Bul. 6, pp. 41-82, 1907.

[36]Fischer, E. Einfluss der Configuration auf die Wirkung der Enzyme. Ber. d. deutsch. chem. Ges. 1894. 27:2985, 3479; 1895. 28:1429. cited by Bayliss, W. M. The nature of enzyme action. 2nd edition, 1911, p. 85. Longmans, Green and Co., New York.