

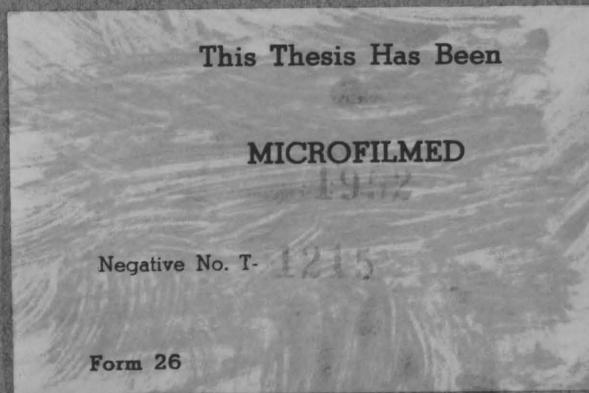
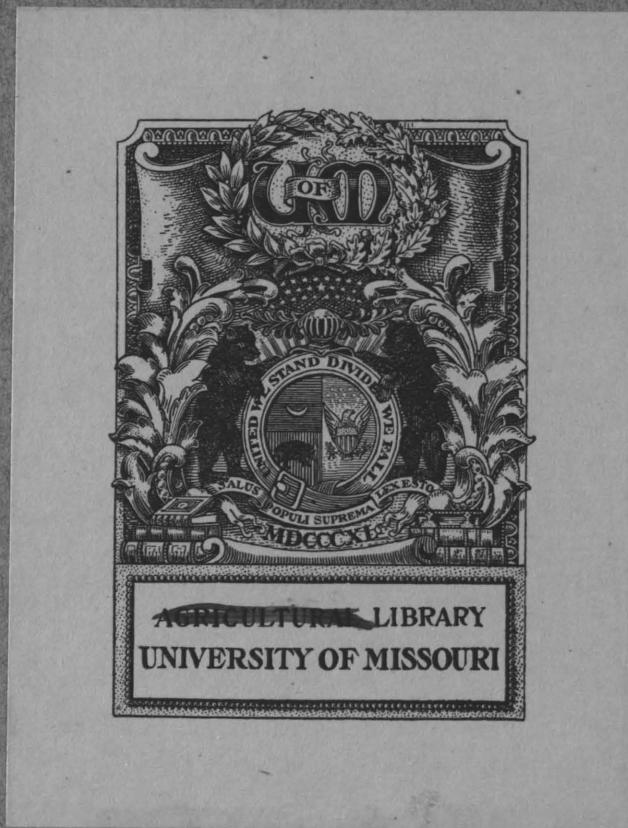


103314908010

Roy Lee Primm.

The efficiency of certain methods of preserving
milk samples for chemical analysis with special
reference to the protein constituents.

This thesis is subject to reserved book regulations.



Primm.

A T H E S I S

THE EFFICIENCY OF CERTAIN METHODS OF PRESERVING
MILK SAMPLES FOR CHEMICAL ANALYSIS WITH SPECIAL
REFERENCE TO THE PROTEIN CONSTITUENTS.

by

ROY LEE PRIMM, A.B.

SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE
MASTER OF ARTS

in the

GRADUATE DEPARTMENT

of the

UNIVERSITY OF MISSOURI

1911.

Approved - Matthew Steel
C. H. Calkles

378.7MTI
XP936

THE EFFICIENCY OF CERTAIN METHODS OF PRESERVING
MILK SAMPLES FOR CHEMICAL ANALYSIS WITH SPECIAL
REFERENCE TO THE PROTEIN CONSTITUENTS.

TABLE OF CONTENTS.

	page
I. Introduction. - - - - -	1
II. The Preservatives selected for test - -	3
a. Formaldehyde - - - - -	4
b. Mercuric Chloride - - - -	8
c. Potassium Bi-chromate - - -	9
d. Thymol - - - - -	10
e. Toluol - - - - -	10
f. Cupric Sulphate - - - -	11
III. Method of testing the Efficiency of the Preservatives - - - - -	11
Analysis - - - - -	12
a. Chemical Methods - - - - -	12
Total Nitrogen - - - - -	12
Casein Nitrogen - - - - -	12
Albumin Nitrogen - - - - -	13
Caseose and Peptone Nitrogen -	14
Residual Nitrogen - - - - -	14
b. Bacteriological Methods - - - - -	14
IV. Experimental work - - - - -	14
a. Experiment I, Preliminary Experiment	14
b. Experiment II Effect of Temperature on Preservative Action.	15

6068₅₉

	page
c. Experiment III. - - - - -	17
a. Effect of Compounding Preservatives.	
b. Effect of Light on Preservative Action	
d. Experiment IV. Uniformity of Results Under Carefully controlled Conditions.	19
e. Experiment V. Effect of the Condition of the Milk at the Time of Adding the Pre- servative on Preservative Action - - -	23
V. Discussion of Results - - - - -	23
a. In general - - - - -	23
b. With Formaldehyde - - - - -	26
c. With Mercuric Chloride - - - - -	28
d. With Potassium Bichromate - - - - -	30
e. With Cupric Sulphate - - - - -	31
f. With Thymol - - - - -	31
g. With Toluol - - - - -	31
h. Complex Preservatives - - - - -	32
VI. Summary of General Conclusions - - - - -	32
VII. Literature cited - - - - -	34

Introduction.

The need has long been felt for an efficient preservative for milk samples intended for chemical analysis. Since it is not feasible to make all analyses on the fresh sample some means must be used to preserve the milk. The method used should make it possible for a correct determination to be made of each constituent. Considerable work has already been reported along this line but the knowledge available on the subject is by no means sufficient or complete. The present investigation was undertaken with the hope of contributing something to the knowledge of this subject. Since the entire subject of milk preservation for chemical analysis is broader than could be undertaken in the time available, the investigation was limited to the preservation of the proteins.

Preservatives Selected -- The preservatives chosen for comparison were formaldehyde, mercuric chloride, potassium bichromate, thymol, toluene and cupric sulphate. The important characteristics of each of these preservatives so far as they have been found in the literature are given below.

Formaldehyde -- Formaldehyde, tho only slightly toxic to certain forms of protoplasm, has been found, in general, to be quite poisonous to the lower organisms. Price (1) found that at ordinary temperatures formaldehyde 1/1500 kills the more common bacteria found in milk; Rivas (2) states that formaldehyde 1/1000 killed all the bacteria in certain milk samples; Clark (3) found that formaldehyde in dilution 1/512 molar was fatal to Aspergillus and Penicillium in beet infusion medium. The germicidal effects of formaldehyde are probably due largely (4,5) to the action of the poison on the proteins of the organism.

Cochran (6) and Lowenstein (7) found that formaldehyde had a marked retarding action on the activity of certain milk enzymes. Beven (8) had some years previously pointed out that the value of formaldehyde as a preservative depended on the condition of the milk at the time of treatment. He reported that if considerable decomposition had taken place formalin did not entirely check decomposition, but merely retarded it. Seligman (9) also found the initial condition of the milk to be an important factor in using formaldehyde as a preservative. Price (1) found that formaldehyde in dilute solution did not have an appreciable influence on the activity of certain proteolytic ferments. This stands in marked contrast to the work of Bandini (10). But of most significance is the finding of Sherman and Tice (11). They have shown that

at 20° C or above, formaldehyde acts merely as a retarding agent to proteolytic enzyme action, and does not check the latter completely, even in the case of milks treated with a preservative soon after milking and kept in air-tight containers. The action of oxidizing ferment of milk is accelerated by formaldehyde according to Seligman (9).

Any unfavorable action formaldehyde may have on enzymes may possibly be attributed to the action of formaldehyde with the compound on which the enzyme acts rather than to an action on the enzyme itself. This view is supported by the work of Herber (12). That formaldehyde does react with protein, altering certain of its chemical and physical properties was first recorded by Trillat (4). Schwarz (13) likewise showed that dilute solutions of albumin on treatment with formaldehyde, especially in the absence of salts, lose their coagubility on treatment with formaldehyde, while more concentrated solutions, on the contrary, under the same treatment retain their coagulability in the presence of salts. Benedicti (14) found the reaction of formaldehyde with proteins to be a slow one, the maximum amount of formaldehyde as a rule, not entering into combination until after two or three weeks. This last is a very strong argument in favor of the hypothesis that the retardation of rennet proteolytic enzymes by formaldehyde is due to the form-

ation of compounds with proteins; for, as Bandini (10), has shown, the retardation of rennet action depends on the length of time the formaldehyde has been (standing) in contact with the milk and on the amount added.

Probably the same reaction to which formaldehyde owes its preservative qualities is responsible for an anomaly very unfavorable for its use as a milk preservative at ordinary temperatures. Cavanaugh (15) attributes to such a reaction the low results obtained for albumin in milk preserved with formaldehyde. Patrick (16,17) called attention to the fact that formaldehyde in concentrations sufficient to prevent appreciable proteolysis caused, in the ordinary course of analysis, an apparent increase in the amount of casein at the expense of the albumin.

Concerning the effect of temperature on the preservative action of formaldehyde in milk but little data was found. Leenhouts (18) called attention to the fact that milk was best preserved with formaldehyde at low temperatures. Summerfield (19) found the germicidal power of formalin to be greatest at $10^{\circ} - 15^{\circ}$ C, however he found it to be equally great at low temperatures if the initial bacterial content was small. He reported that the retardation of the coagulation of milk preserved with formaldehyde at 37° C was inappreciable. This stands in marked contrast to the results of (11) Sherman and Tice and of Jensen (20). They found that milk digested about as

rapidly at room temperature as at 35° C., "apparently", in the words of Sherman and Tice, "because the stimulation of the galactase by the higher temperature is counterbalanced by the fact that formalin affects the enzymes more injuriously in warm than in cold solution".

Seligman (9) discovered that formaldehyde one part to five thousand of milk exerted an elective action on organisms preventing to a greater extent the development of lactic acid bacteria than of other forms. Schaps (9a) found staphylococcus ^{pyogenes} ~~pyogenes~~ more to be resistant than lactic acid bacteria to this preservative. Sherman and Tice (11) found that formaldehyde in concentrations much greater than this, in the case of samples kept at 20° C or above, permitted very little lactic fermentation but quite appreciable proteolytic action.

The rate of disappearance of formaldehyde in milk has been studied by Rivas (2). He recorded that formaldehyde in dilutions one part to ten thousand disappeared from milk in about five days, and that in dilution one part to a thousand an appreciable amount had disappeared in twenty-five days. He found further that the rate of disappearance was more rapid at high than at low temperatures. These results have been confirmed by Williams and Sherman (21) who attribute the disappearance of formaldehyde to its actual destruction.

Mercuric Chloride -- Mercuric chloride has long been recognized as a powerful germicide. Davenport (22) has shown that one seventy thousandth molar concentration in nutritive bouillon prevented the development of spleenic fever bacteria. Clarke (3) found it to be the most toxic agent toward certain fungi, one four thousandth molar concentration proving fatal to Penicillium and Aspergillus. Gerber (23) has made an extensive experimental study of the effect of this salt on the activity of rennets of animal and vegetable origin. He found that it had accelerating action in very dilute solutions, ("due probably to the hydrochloric acid set free by the dissociation of mercuric chloride") and a retarding action in stronger ones. He concludes from his work that mercuric chloride inhibits the activity of the enzyme not by virtue of any direct effect upon the latter, but by forming a complex with the casein which is not readily broken down.

Mercuric chloride on standing in contact with an organic substance is readily reduced to mercurous chloride which is not very poisonous. Perhaps it is to this reaction that mercuric chloride owes most of its antiseptic properties. However, one could hardly hope under ordinary conditions to keep milk sterile for any great length of time unless large amounts of the salt were added or the containers not opened after adding the preservative. Patrick (16, 17) has pointed out that large amounts of mercuric chloride cause precipitation of other protein matter along with the casein.

Potassium Bichromate -- In some countries potassium bichromate is considered the best milk preservative. The French government requires that milk samples which are used for judicial purposes be preserved with this agent. Lindet (24) concluded from numerous communications received from analysts of different countries that potassium bichromate five tenths gram to the litre or sixty drops of formalin to the litre were the preservatives most fit for milk samples to be used for analytical purposes. In this country this preservative has not met with much favor. Woll and Olsen (16, 17), Patrick and Trescot (16,17), and Kenny (17) found it unsatisfactory for the preservation of samples for the determination of casein and albumin nitrogen.

Clarke (3) found that potassium bichromate is almost as fatal to certain moulds as mercuric chloride. Gerber (25) tested the effect of chromium salts and chromates on rennets from several sources. He found that in certain types chromous salts accelerate rennet activity. In other types small amounts were found to retard, and larger ones to accelerate slightly. Neutral chromates were found to retard in all proportions, increasingly so in large doses, checking completely in certain types of rennet. Dichromates were found to retard in certain types of rennin. In other types they retarded in weak doses, but accelerated in large ones. Some relation of these facts as to the efficiency of potassium bichromate as a milk preservative becomes apparent

if it is borne in mind that rennet activity implies peptic activity; and that the presence of widely different types of organisms in milk may mean the presence of proteolytic enzymes of different characteristics as regards their behavior toward the preservative.

Gascard (26) studied the relation of light to the preservation of milk samples with potassium bichromate. He reports having preserved milk perfectly with this agent, five grams to the litre at 12° - 20° C when the milk was kept in a dark place. He found potassium dichromate one part to a thousand not altogether satisfactory even in the dark. In the light, concentrations as high as five parts to a thousand were found very unsatisfactory.

"Kuhn (27) found that potassium dichromate is a better preservative at low temperatures than at high ones.

Thymol -- This compound has been used extensively as a preservative of feces and urine, but very little mention is made in the literature of its properties as a milk preservative. Richmond (28) states that it "keeps" the milk but allows the cream to rise to the surface where it sets in a firm layer and is not readily redistributed. Seligman (9) found thymol to have no influence on the oxidizing fermenters of milk.

Toluol -- In view of the extensive use of toluol in autolytic experiments it was not expected that this agent would protect milk from enzymatic action. Seligman (9)

reports that toluol accelerates the activity of the oxidizing ferments of milk.

Cupric Sulphate -- Concerning the use of this preservative also very little data could be gathered from the literature. Windisch (29) found that Cupric ammonium sulphate, when added in the proportion of one part to a thousand, preserved the 'normal characteristics' of milk for four weeks. Clarke (3) found that copper sulphate one part to a thousand effectually inhibited the germination of penicillium spores altho a molar concentration was required to kill them. But De Seynes (3) grew types of Penicillium in solutions containing from twenty to ninety-five parts of copper sulphate to the litre; and Pfeffer (31) found this mould growing on a concentrated solution of copper sulphate. Gerber (32) found cupric sulphate more marked than mercuric salts both in its accelerating and in its retarding action on various rennets.

Method of Testing the Efficiency of
the Preservatives.

The first steps toward solving the problem in hand was of course that of adopting a proceeding by which the efficiency of the respective preservatives was to be tested. After carefully considering the problem, we decided to approach it from both a chemical and a bacteriological standpoint. This was done in order to learn the condition of the milk at the time of adding the preservative, to differentiate

the effects due to enzymes from those due to living organisms, and in addition, in order to identify any of the latter that might resist a given preservative.

Analysis -- In the analysis of the preserved milk the samples were thoroughly mixed at the temperature at which they were stored, and then the sub-samples were taken for analysis. All determinations were performed as rapidly as possible in order to prevent any further change. With one or two exceptions the result recorded for each determination is the average of two to four closely concordent figures.

In a few cases the acidity of the milk was determined. This was done by titrating the milk directly against normal sodium hydroxide, using phenolphthalein as indicator.

Chemical Methods -- Total Nitrogen -- This is a modification of Hawk's (33) method for total nitrogen in urine. Ten cubic centimeters of milk at 23° C was put in a Kjeldahl flask and digested with twenty-five cubic centimeters of sulphuric acid, 2/10 grams of powdered copper sulphate and six grams of sodium sulphate. The distillation was carried out in the usual way, powdered pumice stone being used to prevent frothing and bumping.

The methods used for the determination of casein and albumin are modifications of those of Van Slyke and Hart, (34).

Casein Nitrogen -- 10 cc of the sample were put

into a clean beaker, warm water was added until the temperature of the milk solution was 44° - 46° C. The temperature of the water added was adjusted so that the total volume when this point was reached, was about 140 cc. Approximately 14 cc. of 1.5% acetic acid were then added from a burette, the contents of the beaker being stirred the while. The precipitate after settling was filtered onto a Munktell's No.0.B. filter paper and washed with .15% acetic acid. The filter paper and precipitate were then thrown into a Kjeldahl flask and the nitrogen determined as in the case of total nitrogen. The reasons for departing from the usual procedure of washing the precipitate with dilute acid instead of water were threefold -- First: The casein of certain preserved samples was found to be so altered when it was washed with water that it ran thru the filter paper. Satisfactory results were obtained when the casein was washed with acetic acid of the above concentration. Second: The distilled water furnished to the laboratory was slightly alkaline due to dissolved lime, Third: Bechamp (35) has pointed out that casein is soluble even in pure water.

Albumin Nitrogen -- The filtrate from the casein, which in every case amounted to about 225 cc. was exactly neutralized with sodium hydroxide (Phenolphthalein was used as indicator) and 2 cc. of 1.5% acetic acid added. The solution was then raised quickly to the boiling point and

boiled for four minutes. The precipitate was treated in a manner similar to that of the casein, except that it was washed with water instead of dilute acid.

Caseoses and Peptones -- The filtrate from the albumin was treated with 15 cc. Almen's tannic acid solution and stirred. This precipitate was then treated in a manner similar to the precipitated albumin.

Residual Nitrogen -- The filtrate from the caseoses and peptones was poured into a Kjeldahl flask and its nitrogen content determined as in the case of total nitrogen.

Bacteriological Methods -- The methods used in the bacteriological analyses were essentially those given in texts on the subject. The media employed for the isolation and counting of the bacteria (and moulds) were peptone-milk-serum agar and milk-serum gelatin. Thruout most of the work only agar plates were made, slope cultures were made of predominating types of colonies and kept for future study. In a few instances some of the cultural and morphological characteristics were worked out but in most cases the qualitative analysis has not yet been completed. The subsamples used for bacteriological analysis were taken at the temperature (approximately) at which the original samples were preserved.

Experimental Work.

Experiment I. -- A large sample of milk, the condition

of which is indicated by the analyses, was put thru the cream separator. The skim milk was poured into a large carboy and thoroughly mixed. Samples were then removed and treated with preservatives as indicated in Table I. The milk was well shaken just before withdrawing each sample. This precaution was taken in all experiments. The samples were preserved in rubber stoppered bottles of approximately five hundred cubic centimeters capacity. Part of the samples were put in a dark locker at 18° - 25° C. and part were put in a dark cooling room at 8° C. Immediately after taking the samples to be preserved, a sample of the milk was taken for bacteriological and mycological analysis and another sample was taken for chemical analysis. The plates for the bacteriological analysis were made as soon as possible and put in an incubator at 25° C. The chemical analysis was then started. The bacterial count for the milk at the time of sampling was 400,000,000 per cc. The chemical analysis gave

Total nitrogen - - - - - .583 %

Casein nitrogen - - - - - .447 %

Albumin nitrogen - - - - - .059 %

Caseose and Peptone nitrogen - .049 %

The treatment of the preserved samples and the results of the analysis of same are indicated in Tables I and 2.

Experiment II. -- This experiment was performed primarily to confirm the results of the previous experiment.

Table I

Number of Sample	Preservative		Temperature	Casein Nitrogen and Albumin Nitrogen				Casein Nitrogen %		
	Kind	Concentration		Just Before Sampling		28 Days after Sampling				
				Casein Nitrogen %	Albumin Nitrogen %	Casein Nitrogen %	Albumin Nitrogen %			
A1	HgCl ₂	1/1000	80°	.447	.059	.422	.058	.347		
A2	HgCl ₂	1/2000	"	. "	. "	.395	.047	.324		
A3	Thymol	1/1000	"	. "	. "	.390	.036	.		
A4*	HCHO	1/5000	"	. "	. "	.392	.031	.		
A5†	HCHO	1/5000	"	. "	. "	.395	.049	.		
A6‡	Thymol	1/1000	"	. "	. "	.394	.036	.335		
A7	HCHO	1/5000	"	. "	. "	.382	.032	.		
A8	HgCl ₂	1/2000	"	. "	. "	.361	.043	.242		
A9§	Thymol	1/1000	"	. "	. "	.348	.035	.		

* Coagulated about 40 days after sampling.

† " " 43 " " " " ;

‡ " " 35 " " " " ;

§ " " 30 " " " " ;

Table II

Number of Sample	Mould Spores and Bacteria per Cubic Centimeter							
	Just before adding Preservative		7 Days after taking Sample		23 Days after taking Sample		74 Days after taking Sample	
	Mould Spores	Bacteria	Mould Spores	Bacteria	Mould Spores	Bacteria	Mould Spores	Bacteria
A1	0	400,000,000	0	0	0	0	14,000	14,000
A2	"	"	0	35,000	0	0	7,050	52,000
A3	"	"					0	700,000
A4	"	"	0	4,000			80	4,000,000
A5	"	"						
A6	"	"	0	2,000,000			0	600,000
A7	"	"	0	200				
A8	"	"	0	750			5,000	32,500
A9	"	"	0	2,000,000				

The Relation of Temperature to Preservative Action.

Fifty litres of morning's milk were poured soon after milking into a large carboy and stored in a cooling room at 3° C for nine hours. The carboy was then taken to the sampling room (temperature 16° C) where the sampling was done in a manner similar to that of Experiment I. Table III indicates the treatment of the samples and the results of analyses. Samples 6, 7, 12, and 15 were preserved in 2.4 litre glass stoppered bottles and the others of this series were preserved in rubber stoppered bottles of approximately 3 litre capacity. The number of bacteria in the milk at the time of sampling was 5,000,000 per cc. No mould spores were present. The chemical analysis gave:

Total nitrogen - - - - -	.548 %
Casein nitrogen - - - - -	.422 %
Albumin nitrogen - - - - -	.056 %
Caseose and Peptone nitrogen - -	.039 %
Residual nitrogen - - - - -	.028 %

Experiment III -- The primary objects of this experiment were to find the effect of weak diffused light, and the effect of compounding preservatives on preservative action. About fifty litres of milk in two eight gallon cans were put soon after milking in a 3° C cooling room. After seven hours (approximately) the milk was taken to the sampling room and thoroughly mixed in a large

Table III
Showing Relation of Temperature to Preservative Action.

Number of Sample	Preservative		Temperature	Casein Nitrogen		Physical Condition of the milk 40 days after Sampling	Mould Spores and Bacteria per c.c		
	Kind	Concentration		Days after Sampling	%		Days after Sampling	Mould Spores	Bacteria
M ₀	No Preservative		20°-30°	0	.422	Mixable only	0	0	5,000,000
M ₁	HCHO	1/2500	20°-30°	40	.393	Mixable only after warming	15	3,000	310,000
M ₂	HgCl ₂	1/1000	"	"	.398	"	"	3,800	0
M ₃	K ₂ C ₂ O ₇	1/1000	"	"	.309	"	"	18,000	625,000
M ₄	HgCl ₂	1/2000	"	"	.341	"	"	1,100	160
M ₅	HCHO	1/2500	"	"	.	"	"	-	-
M ₆ [*]	Thymol	1/670	"	"	.		"	0	12,000,000
M ₇ [†]	HCHO	1/5000	"	"	.		"	25,000	21,150,000
M ₈	K ₂ C ₂ O ₇	1/1000	2°-30°	"	.401	Readily mixed in the cold	"	10	0
M ₉	HgCl ₂	1/2000	"	"	.401	"	"	0	0
M ₁₀	HCHO	1/1250	"	"	.433	"	"	0	140
M ₁₁	HCHO	1/2500	"	"	.423	"	"	8	7
M ₁₂	HCHO	1/5000	"	"	.363	"	"	10	240
M ₁₃	HCHO	1/1250	"	"	.438	"	"	0	10
M ₁₄	HgCl ₂	1/1000	"	"	.421	"	"	0	0
M ₁₅ [‡]	Thymol	1/670	"	"	.		"	0	12,000,000

* Coagulated about the 3rd day after sampling.

† " " " " 5th " " "

‡ " " " " 7th " " "

carboy. Samples were taken as in the preceding experiments. The samples were preserved in rubber stoppered bottles of 500 cc. to 700 cc. capacity, excepting samples Na6, Nb5, and Nb7, which were preserved in bottles of 940 cc. 1440 cc. and 3600 cc. capacity, respectively. The treatment of the preserved samples and analytical results are recorded in Tables IV and V. The milk contained at the time of sampling 1,353,000 bacteria per cc. and no mould spores. The chemical analysis was as follows:

Total nitrogen - - - - -	.530 %
Casein nitrogen - - - - -	.405 %
Albumin nitrogen - - - - -	.058 %
Caseose and Peptone nitrogen -	.045 %
Residual nitrogen - - - - -	.028 %

Experiment IV.-- This experiment was performed for the purpose of testing the degree of uniformity in the behavior of duplicate samples subjected as nearly as practicable to the same conditions. Twenty-five litres of milk were strained thru a thick towel immediately after milking. The milk was taken to the sampling room and samples taken as in the last experiment. The treatment and results of analyses of samples of this set are recorded in Table VI.

Table IV

Showing Effect of Compounding Preservatives.

Number of Sample	Preservative		Temperature	Chemical Analysis		Mould Spores and Bacteria per cc	
	Kind	Concentration		Days After Sampling	Casein Nitrogen	Days After Sampling	Mould Spores
N _a ⁰	No Preservative		0	.405	0	0	1,353,000
N _a ¹	HgCl ₂ K ₂ C ₂ O ₇	1/1000 1/1000	8°C 37	.375	26	0	0
N _a ²	HCHO	1/2500	" "	.380	"	1,700	3,100
N _a ³	HCHO Thymol	1/2500 1/2000	" "	.419	"	1,900	680
N _a ⁴	HgCl ₂ Thymol	1/1000 1/2000	" "	.412	"	0	0
N _a ⁶	K ₂ C ₂ O ₇ HgCl ₂	1/1000 1/2000	" "	.373	"	0	0
N _a ¹⁰	Thymol HgCl ₂	1/2000 1/2000	" "	.383	"	80	0
N _a ¹¹	K ₂ C ₂ O ₇ HCHO	1/1000 1/2500	" "	.386	"	60	20
N _a ¹³	HgCl ₂ HCHO Thymol	1/4000 1/5000 1/2000	" "	.435	"	0	0
N _a ²¹	HCHO HgCl ₂	1/2500 1/1000	" "	.401	"	10	0
N _a ²²	HCHO HgCl ₂	1/5000 1/500	" "	.431	"	3,000	0

Note:— All of the samples of this series were readily mixed in the cold.

Table V
Showing Effect of Diffused Sunlight on Preservative Action.

Number of Sample	Preservative		Container	Temperature	Chemical Analysis					Mould Spores and Bacteria per cc		
	Kind	Concentration			Days After Sampling	Casein Nitrogen %	Albumin Nitrogen %	Casein and Peptone Nitrogen %	Residual Nitrogen %	Days after sampling	Mould Spores	Bacteria
N ^b ⁰	No Preservative				0	.405	.058	.045	.028	0	0	1,35,000
N ^b ¹	K ₂ Cr ₂ O ₇	1/200	Brown Bottle	17°C	28	.370	.042	.085	.030	26	10	10
N ^b ²	K ₂ Cr ₂ O ₇ Thymol	1/1000 1/2000	White Bottle	"	"	.377	.057	.064	.032	"	0	0
N ^b ⁴	HCHO	1/2500	White Bottle	"	"	.394	.033	.067	.033	"	90	0
N ^b ⁵	HCHO	1/2500	Blue Bottle	"	"	.385	.031	.076	.033	"	4,200	180
N ^b ⁷	HCHO HgCl ₂	1/2500 1/2000	Blue Bottle	"	"	.403	.029	.064	.031	"	0	0
N ^b ⁸	K ₂ Cr ₂ O ₇	1/1000	White Bottle	"	"	.321	.072	.087	.041	"	28,000	40,000
N ^b ¹⁰	K ₂ Cr ₂ O ₇	1/1000	Brown Bottle	"	"	.346	.065	.084	.040	"	1,600	38,000
N ^b ¹¹	K ₂ Cr ₂ O ₇	1/200	White Bottle	"	"	.368	.046	.087	.023	"	0	40

Note:- None of the samples of this set were very readily nor yet very difficultly mixed.

Sample N^b 8 coagulated about the 35th day after sampling.

Table VI

Showing Degree of Uniformity of Results under Carefully Controlled Conditions.

Number of Sample	Preservative		Temperature	Chemical Analysis						Mould Spores and Bacteria per cc		
	Kind	Concentration		Days After Sampling	Total Nitrogen%	Casein Nitrogen%	Albumin Nitrogen%	Caseose and Peptone Nitrogen%	Residual Nitrogen%	Days after Sampling	Mould Spores	Bacteria
D ₀	No Preservative			0	.539	.418	.056	.038	.029	0	0	550
D ₁	HgCl ₂	1/2000	2°C	17	.539	.407	.065	.034	.032	34	0	0
D ₂	HgCl ₂	1/2000	"	"	.537	.407	.068	.034	.032	"	30	0
D ₃	HCHO	1/2500	"	"	.540	.420	.051	.039	.028	"	20	0
D ₄	HCHO	1/2500	"	"	.538	.420	.051	.039	.026	"	130	0
D ₅	Thymol	1/5000	"	"	.540	.394	.073	.049	.029	"	5,000	12,000,000
D ₆ [*]	Thymol	1/5000	"	"	.540	.393	.073	.049	.029	"	600	10,000,000
D ₇ [#]	Toluol	1/200	"	"	.540	.394	.069	.049	.029	"	0	7,500,000
D ₈ [#]	Toluol	1/200	"	"	.539	.391	—	.051	.029	"	0	6,000,000

* Coagulated about 8 days after sampling. # Coagulated about 17 days after sampling. + Coagulated about 10 days after sampling.

Experiment V -- The chief end in view in performing this experiment was the determination of the relation existing between the preservative action of an agent and the condition of the milk at the time of adding the preservative. Fifty litres of milk were taken to the sampling room as soon as it came from the barn. The bulk of the milk was placed in a carboy and thoroughly mixed. The sample was then divided into two parts. Samples were taken from one part immediately. The treatment and analyses of this set of samples are indicated in Table VII. The other part was allowed to stand in the sampling room (temperature 17° C) for 40 hours. Samples were then taken from this portion. The treatment and analyses of this set of samples are recorded in Table VIII.

Discussion of Results.

The Preservatives in General -- Most of the results will be discussed under the head of the individual preservatives. However, there are a few remarks applicable to all or a number of the preservatives which can appropriately be made here. In the first place, most of the preserved samples which had stood forty days or longer seemed to show some alteration in the casein. This change was manifested by the behavior of the casein in the course of analysis. Whereas the casein of fresh milk was almost entirely retained on the filter paper on washing with water,

Tables VII and VIII

Showing the Relation of the Condition of the Milk at the time of Adding the Preservative to Preservative Action.

Table VII

Number of Sample	Preservative		Temperature	Chemical Analysis						Mould Spores and Bacteria per c.c.		
	Kind	Concentration		Days after Sampling	Total Nitrogen %	Casein Nitrogen %	Albumin Nitrogen %	Caseose and Peptone Nitrogen %	Residual Nitrogen %	Days after Sampling	Mould Spores	Bacteria
E ₀	No Preservative		8°C	0	.500	.380	.055	.037	.029	0	0	1,200
E ₃	K ₂ Cr ₂ O ₇	1/200	8°C	31		.357	.032	.074	.029	22	0	0
E ₄	K ₂ Cr ₂ O ₇	1/400	"	"		.356	.048	.064	.029	"	10	0
E ₇	HCHO	1/2500	"	"		.367	.043	.053	.032	"	400	0
E ₅	CuSO ₄	1/350	"	"		.381	.	.	.	"	20	20

The milk referred to in table VII was sampled while fresh; that referred to in Table VIII was sampled after standing 40 hours at 17°C. The two milks were taken from the same original sample.

Table VIII

E _{X0}	No Preservative	"	0	.500	.358	.062	.048	.029	0	0	3,000,000,000
E _{X1} [*]	HCHO 1/2500	"	29		.351	.034	.059	.053	20	0	0
E _{X2} [*]	HCHO 1/650	"	"		.349	.031	.061	.055	"	0	0
E _{X3}	HgCl ₂ 1/2000	"	"		.309	.051	.071	.067	"	0	50,000
E _{X4}	K ₂ Cr ₂ O ₇ 1/200	"	"		.338	.048	.065	.047	"	0	0
E _{X6}	HgCl ₂ 1/1000	"	"		.359	.060	.033	.051	"	0	0

* Coagulated about the 10th day after sampling.

a large per cent was found to pass thru in the case of the preserved samples. This was especially true of the samples of series A at the time of making the last casein analysis recorded in Table I. The same was true in a lesser degree of all samples preserved, save possibly those treated with potassium dichromate. As has been mentioned previously, washing the casein with .15 % acetic acid instead of water prevented this peculiar passage of the casein thru the filter paper.

All the preservatives showed greater enzyme retarding action at low temperatures than high. No exception to this statement was found. The germicidal power of the preservatives also seemed to vary inversely with the temperature at which the samples were kept. The physical condition of the samples was found to be uniformly better when kept at a low temperature than when held at room temperature or above.

Although most of the preservatives showed a very marked germicidal power on bacteria none of them entirely prevented the development of moulds, especially if there was plenty of air space above the surface of the preserved milk. In case the samples were kept at low temperature the mould growth was greatly retarded, but even at 2° C some mould growth occurred if plenty of air was available. The proteolytic effect of equal amounts

of mould growth was greater at room temperature than lower ones. The proteolytic effects of moulds on the preserved samples were greater than those of bacteria. The most important factor in proteolysis of preserved samples at low temperatures seems to be the proteolytic enzyme originally in the milk. A glance at Table VIII will show this fact very clearly.

Formaldehyde -- This preservative in concentrations 1/5000, was very unsatisfactory when the milk was preserved at room temperature, or at 2° C. At 2° C. it was found to be satisfactory in concentration 1/2500 even when the milk had a high bacterial content at the time of adding the preservative. At 8° C. or above proteolysis was not prevented by concentrations as high as 1/1670 regardless of the condition of the milk. (Bacteriologically speaking). Formaldehyde in concentration 1/1650 did not have any greater retarding effect on proteolysis, on hydrolysis of the sugar, or on coagulation of the milk than in concentration 1/2500. In samples Experiment 1 and Experiment 2 which had a high initial bacterial content, the lactic acid increased some 30% in the first 40 days after adding the preservative. This indicates that the initial condition of the milk is an important factor in the preservation of the milk sugar with formaldehyde.

A marked increase in the apparent casein content was noted in Samples M 10 and M 13 which were preserved with formaldehyde in 1/1250 at 2° C.

This phenomenon is hard to explain on the basis of a direct reaction of the formaldehyde with the albumin and globulin, but is readily explained on the assumption that casein, albumin, and globulin exist in milk as a complex and this complex is so affected by formaldehyde that less albumin and globulin are split off in making an analysis than is the case with milk not treated with formaldehyde. Gerber (12) and Vandevelde (36) working along different lines found strong evidence that casein, albumin and globulin exist as a complex in raw milk. Whatever may be the explanation for the apparent increase in casein nitrogen when formaldehyde is used as a preservative, my results confirm those of Patrick (16,17) Woll and Olsen (16,17), and Kenney (17).

The importance of temperature in preserving milk with formaldehyde is well illustrated by comparing the results of sample E7 with those obtained for sample M 11. The latter milk contained 4,000 times as many bacteria as the former at the time of sampling; yet, the casein in the latter sample was preserved, while there was a marked falling off in both the casein and albumin in the former. The difference in temperature at which the two samples were kept was only 6° C. ! It will be noticed that sample E7 contained a considerable growth of mould, and this may account in part for the proteolysis which had taken place. This view is further fostered by the fact that sample E7

showed almost as much proteolysis after 30 days as samples Ex1 and Ex2. The absence of mould in samples Ex1 and Ex2 is probably to be explained on the assumption that products formed by the bacteria were toxic to the mould. This may partly explain why M11 contained so little growth of mould. However, the difference in the temperature at which the samples were kept is probably the chief factor in the explanation. It was also noted that those samples which had been infected with moulds for some time showed decidedly more proteolysis than those free from mould. In the course of the investigations it was frequently noted that an abundant growth of mould developed in milk preserved with formaldehyde when the container was only partly filled. The tables do not illustrate this fact very well since no record was made as to the length of time mould growth was present before the chemical analysis was made, and further, most of the containers were nearly full until the samples were removed for analysis.

Mercuric Chloride -- This preservative in concentration 1/2000 permitted appreciable proteolysis under all of the experimental conditions tested. The results obtained with samples D1 and D2 are interesting in that they show that albumin may be one of the first cleavage products formed when casein is subjected to the action of certain proteolytic ferments. This in the case of rennin has already been demonstrated by Schmidt and Nielsen (37).

Mercuric chloride in concentration 1/1000 proved eminently satisfactory as a milk preservative if the samples were kept at 8° C. or at a lower temperature. (Samples M14 and Ex16). Sample A1 is an exception. This may be accounted for partly by the fact that it was brought to room temperature several times and possibly partly by the particular enzymes from the dead and living bacteria. The fact that the milk was skimmed may also be significant. It should be noted in this connection that the milk used by most investigators for testing the protein preservative action of various chemicals was skimmed milk, whereas the milk most generally used for analysis is whole milk.

Samples A1 and A2 show a falling off in bacterial numbers to less than 1 organism per cc. and then a gradual rise in number of organisms. Both samples also show appreciable mould growth. It will be noted that the rate of proteolysis is greater during the period from the 28th day to the 79th day than during the period from the first to the twenty-eighth day. The period of increase in rate of proteolysis coincides with the period of increase in number of organisms. The checking of proteolysis after considerable of the casein has decomposed is illustrated by sample A8. While there is appreciable mould growth and even some bacterial increase in the second period the rate of proteolysis is lower in the second period

than in the first. This is probably to be explained on the basis that the decomposition products of the protein exerted an inhibiting action on the proteolytic enzymes. The fluctuation in room temperature may also have had some influence on this action.

The Remarks concerning mould contamination of samples preserved with formaldehyde are equally applicable in the case of samples preserved with mercuric chloride.

Potassium Bichromate -- This preservative was not found satisfactory under any circumstances. Proteolysis was several times greater in samples kept at room temperature than in samples kept at low temperature. For instance, the proteolysis in Sample M8 at 2° C. was, in the same length of time, but 1/6 as much as that in Sample M3.

Mould growth was far more abundant at room temperature than it was at lower temperatures. This accounts for much of the proteolysis that occurred in sample M3. It is interesting to note that potassium dichromate permitted as much proteolysis when added to the milk, while fresh, as when added to the milk after the bacterial content had reached 3 billion bacteria per cubic centimeter. (Compare result on Sample E3 with that on Sample Ex4.)

A comparison of the results on sample Eo with those for Exo and those for Ex4 with those for Exo shows that the rate of proteolysis in milk preserved with potassium bichromate at 8° C. was approximately 1/7 as great as that which

had taken place at 17°C . in the milk without preservative.

Diffused light was found to lessen the preservative action of potassium dichromate in concentrations 1/1000 (samples Nb8 and Nb10). Thus far the work of Gascard (26) is confirmed. His claim that potassium dichromate 1/200 in the dark preserves the casein perfectly for a month is not confirmed.

Cupric Sulphate -- Cupric sulphate at 8° C . (sample E5) preserved the casein perfectly for thirty-five days. This chemical has not yet been tested for its preservative effect on the other constituents of the milk.

Thymol -- Thymol was found very unsatisfactory as a milk preservative. In all experiments with unskimmed milk thymol in concentration up to 1/670 failed to prevent coagulation of the milk. In experiment I with skimmed milk at 8° C . it prevented coagulation of two samples for ninety days, but portions of these samples brought to room temperature after this time coagulated in a short time. In this experiment one sample preserved with thymol at room temperature did not coagulate for thirty days.

Thymol 1/5000 at 2° C . did not stop the growth of organisms. In higher concentrations it caused a decrease in the number of bacteria, but did not completely sterilize the samples after a month or longer. Peculiarly enough, however, very few of the milk samples preserved with thymol

alone showed mould growth. In Experiment I, Thymol 1/1000 seemed to inhibit proteolysis in a degree very slightly less than mercuric chloride 1/2000.

Toluol -- This preservative was very much more unsatisfactory than thymol. In concentration 1/200 toluol checked proteolysis as much as thymol in concentration 1/5000.

Complex Preservatives.

By complex preservatives is meant two or more preservatives used in the same sample. More striking results were obtained by the use of complex preservatives than was anticipated. Table IV shows that mercuric chloride 1/1000 plus formaldehyde 1/2500 at 8° C. preserved the casein for thirty days. Mercuric chloride 1/500 plus formaldehyde 1/5000 caused an increase in the apparent casein nitrogen. Thymol 1/2000 plus mercuric chloride 1/2000 was no more efficient than mercuric chloride 1/2000. Thymol 1/2000 plus mercuric chloride 1/1000 caused a slight increase in the apparent casein, whereas thymol 1/2000 plus formaldehyde 1/2500 caused a further increase in the amount of casein,

Summary of General Conclusions.

Preservatives, in general, are most efficient at low temperatures. A few degrees difference in temperature may mean the difference between a good and an inefficient pre-

servative, at least as far as the milk proteins are concerned.

The enzymes in the milk at the time of taking from the udder and moulds were the important factors causing proteolysis of the casein and albumin in the preserved samples. The effects on the casein and albumen of preserved samples due to the enzymes produced by the more common bacteria which developed in milk kept at 18° C. for forty hours were almost insignificant when the samples were kept at low temperatures.

Diffused light is an important factor in preservation of milk with potassium bichromate in weak concentration, but not in the case of formaldehyde.

Mercuric chloride 1/1000 at 2° C. or thereabouts, is a perfect preservative of the casein and albumin for thirty days or longer.

Formaldehyde 1/2500 is an excellent preservative of all protein constituents of milk provided the samples are kept at 2° C. or lower.

Cupric sulphate 1/350 is an efficient preservative of the casein for 40 days at 8° C.

The above preservatives at high temperatures are not satisfactory preservatives.

Potassium dichromate, thymol, and toluol are unsatisfactory as preservatives of milk for a week or longer.

There is possibility of finding a very efficient milk preservative in a very weak concentration of the formaldehyde-thymol-mercuric chloride complex.

This investigation was undertaken at the suggestion of Dr. Matthew Steel, who is in charge of the Dairy Research Laboratory of the United States Department of Agriculture in cooperation with the Department of Dairy Husbandry of the University of Missouri.

LITERATURE CITED.

1. Price, U.S. Dept. Agr. Bureau of Animal Industry Report, 1903, p.114.
2. Rivas, Univ. Penn. Med. Bul. 1904, 17, p.175.
3. Clarke, J.F. Botanical Gazette, 1899, 28, 289 and 278.
4. Trillat, Compt. rend. 1892, 114, 1278.
5. Hauser, Munch. Med. Wochensch. 1903, p.567.
6. Cochran, C.B. Penn. Dept. Agr. Report 1899. p.277.
7. Lowenstein, Ztschr. Hyg. u. Infektionskrank. 1904. 48, p.239.
8. Bevan, E.J., Analyst. 1895. 20. p.152.
9. Seligman, E. Ztschr. Hyg. u. Infektionskrank. 1905. 50. p.97.

- 9a. Schaps, L. Ztschr.Hyg.u. Infectionskrank, 1905.
50, p. 247.
10. Bandini, P. Centrbl.Bakt. (etc.) I Abt. Orig. 1906.
41, pp. 271, 379 and 474.
11. Sherman and Tice, Jour.Am.Chem.Soc. 1906, 28, 189.
12. Gerber, C.Compt.rend. 1910, 150, 1357.
13. Schwarz, Zeit.Physiol.Chem., 1901, 31, 460.
14. Benedicenti. Archiv.f.Anat.u.Physiol. 1897, 219.
15. Cavanaugh, Report of the Referee on Dairy Products
to the 19th Annual Convention of the Association
of Official Agricultural Chemists. 1903.
16. Patrick, Report of the Referee on Dairy Products
to the Twenty-First Annual Convention of the Associa-
tion of Official Agricultural Chemists, 1905.
17. Patrick, Report of the Referee on Dairy Products
to the 22d Annual Convention of the Association of
Official Agricultural Chemists. 1906.
18. Leenhouts, P.Organn. Ver.Oud leer Rijks Landouwschool.
1900. 12, 195.
19. Sommerfeld, P.Ztschr.Hyg.u. Infectionskrank. 1905.
50, 153.
20. Jensen, Jour.Am.Chem.Soc. 1900, 14, 197.
21. Williams and Sherman. Jour.Am.Chem.Soc. 1905. 27, 1497.
22. Davenport, Experimental Morphology, 1897.
23. Gerber, C.Compt.rend.soc.biol. 1910. 68, 631, 636, 765.
24. Lindet. Rev.Gen.Lait. 1903, 370.

25. Gerber.C. Compt.rend.soc.biol.1910.69,215.
26. Gascard. Compt.rend.1909,148,580.
27. Kuhn M. Molk.Ztg.1894.8,354.
28. Richmond, H.D. Dairy Chemistry.1899.
29. Windisch. Milchw.Zentbl.1908,4,97.
30. De Seynes, J.Bull.De la Soc.Bot.de Fr.1895,42;
451 and 482.
31. Pfeffer, W. Pflanzenphysiologie, 2,1881.
32. Gerber, C.Compt.rend.soc.biol.1910,68,768.
33. Hawk, P.B. Jour.Am.Chem.Soc. 1907.29,1634.
34. Van Slyke and Hart. Am.Chem.J.1903.29,150.
35. Bechamp, Bul.Soc.Chim.Paris.1893.11,152.
36. Vandevelde. Biochem.Z.1911.29,461.
37. Schmidt and Nielsen. Beitr.Chem.Physiol.u.Path.
1907.9,322.

University of Missouri - Columbia



010-100933028

378.7M71
XP936

378.7M71
XP936

6068

378.7M71
XP936

