

A QUANTITATIVE STUDY OF THE HEAT LABILE
SULFIDES OF MILK

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by

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A THESIS

Submitted to the Graduate School of Michigan State
College of Agriculture and Applied Science in Partial
Fulfilment of the Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department of Dairy Husbandry

1942

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ACKNOWLEDGMENTS

The writer wishes to express his sincere appreciation to Doctor Earl Weaver, Head of the Dairy Department, for making this study possible, and to Doctor I. A. Gould for his valued guidance in conducting this study and in the preparation of this manuscript.

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INTRODUCTION

The recent trends toward the use of higher temperatures in the processing of dairy products has caused considerable attention to be directed toward the volatile sulfides and sulfhydryl group appearing at such temperatures. These compounds have been shown to be especially significant in heat treated products since they appear rather abruptly at approximately the same temperature found to produce a cooked flavor, prevent an oxidized flavor, destroy certain enzymes and cause a redistribution of nitrogen. In addition, these Sulfide and sulfhydryl compounds are apparently associated with the purple ring formation in evaporated, and perhaps has some significant role in the browning of milk subjected to sterilization temperatures.

The source of the volatile sulfides evolved from milk during heating has been shown by quantitative tests to be the serum proteins and fat globule membrane. However, the conclusions drawn in this regard are not entirely in harmony and a number of the factors influencing the heat liberation of sulfides have not been fully investigated. In addition, the amount of sulfides contributed by each of these constituents and the total amount of sulfides evolved by heat treated milk has not been measured quantitatively.

This study was conducted to determine quantitatively the volatile sulfides of milk and the influence of certain factors on these sulfides. Also, efforts were made to ascertain the relationship between the heat liberated sulfides and the total sulfur content of milk.

HISTORICAL

Heat Formation of Hydrogen Sulfide and Sulfhydryl Compounds

Early observations: Hydrogen sulfide was early identified by Schreiner (136), Neimann (111), Hubner (131), Oppenheimer (113), Rettger (124), and Utz (151), as being among the volatile constituents liberated when milk is subjected to high temperatures. Similar observations have been made more recently by Konig and Schreiber (88). In general, these experiments were concerned with the development of specific tests to differentiate between heated and unheated milk or to demonstrate general decomposition of milk by high temperature treatment.

Lead acetate paper or cotton soaked in lead acetate solution were used by the majority of the early workers. Oppenheimer (113) in 1901, used lead acetate paper for sulfide detection and obtained from 3 to 6 mg. of sulfur by boiling 300 cc. of milk for 30 minutes. He detected no sulfur compounds when the milk was held 30 minutes at 75° C. In a similar manner Utz (151) observed a small amount of hydrogen sulfide liberation from milk heated to near boiling, and points out that another investigator (Schreiner) observed hydrogen sulfide liberation from milk at temperatures between 81 and 82° C. Rettger (124), by suspending cotton soaked in lead acetate solution above heating milk, was unable to detect hydrogen sulfide liberation until the temperature exceeded 85° C. This worker observed that pasteurized milk and condensed milk liberated only small amounts of hydrogen sulfide, and that upon prolonged heating of milk a point was reached at which hydrogen sulfide was no longer evolved.

In 1927, Konig and Schreiber (88) boiled milk 4 hours in an atmosphere of nitrogen and collected the volatile sulfur in a train con-

taining a silver solution. By this procedure they obtained 3.8 mg. of sulfur per liter of milk.

Recent observations: A more recent study on the problem of sulfide liberation in milk is presented by Gould and Sommer (45). These workers used basic lead acetate paper and rather large volumes of milk to show that hydrogen sulfide liberation is a function of time as well as temperature. Sulfides were detected when milk was heated from 76 to 78° C. momentarily, from 74 to 76° C. for 3 minutes, or from 70 to 72° C. for 30 minutes. Positive nitroprusside tests were obtained on milk heated to similar temperatures by Josephson and Doan (75) and Gould (48), indicating the formation of sulfhydryl groups.

Flake, Jackson and Weckel (36) conducted limited quantitative studies on the heat liberation of volatile sulfur. By vacuum distillation at 77 to 79 C. they were able to recover 0.0036 mg. of sulfur from 350 cc. of milk. In other quantitative studies, Diemair, Strohecker and Keller (27) added 6 cc. of 1 per cent sodium hydroxide to 10 cc. of milk and distilled over the volatile sulfur. They report the following values for 100 cc. samples: raw milk, 0.13 to 0.15 mg.; pasteurized milk, 0.11 to 0.14 mg.; and cream, 0.07 to 0.11 mg.

Observed Sources of Hydrogen Sulfide and Sulfhydryl
Compounds and Factors Influencing Their Libera-
tion by Heat

Hydrogen-ion- Concentration: Hydrogen-ion concentration is generally known to influence the stability of proteins and observations on milk have shown this factor to influence hydrogen sulfide liberation by heat. Osborne (114), Zahnd and Clarke (166), Blumenthal and Clarke (15),

Abel and Geilling (1), Brand and Sandberg (18), Jones and Gersdorff (74), and Vickery and White (156) have shown cystine to be unstable to alkali. On the other hand, methionine has been reported to be rather stable to alkali by Mueller (109), Painter and Franke (118), and Masters (97). Shinohara and Kilpatrick (140) found that casein would liberate hydrogen sulfide after heating at 80° C. for 6 hours in an acid medium.

Rettger (124) concluded from his early observations that the liberation of hydrogen sulfide from milk was enhanced by a slight alkalinity and retarded by slight acidity. According to Jackson, Howat and Hoar (69) sodium bicarbonate, disodium phosphate, or sodium citrate added to canned cream at the rate of 5 grams per gallon enhanced sulfide liberation during sterilization, and decreased the temperature and period of heat exposure necessary to give a positive nitroprusside test.

The work of Gould and Sommer (45) indicates that the pH of the sample may influence the lability of sulfur in milk. They observed that milk at a pH of 7.6 formed a cooked flavor at a lower temperature than normal milk, whereas a pH of 5.8 to 6.0 retarded cooked flavor formation. In other work Gould (48) states that whey has a normal critical temperature even when heated at pH 4.6.

Fat percentage: One may logically conclude that increases in fat percentage of a sample of milk would decrease the amount of available sulfur since there would be a corresponding decrease in the protein content. In quantitative studies, Rettger (124) and Diemair, Strohecker and Keller (27) report that skim milk liberates more hydrogen sulfide and cream less hydrogen sulfide than normal milk. In contrast, Gould and Sommer (45) and Gould (48) demonstrate that increases in butterfat decrease the temperature

at which hydrogen sulfide appears. Skimmilk required a somewhat higher temperature than milk before evolving sulfides, and cream, buttermilk whey liberated sulfides at a lower temperature than milk. The following critical temperatures for a 3 minute heating period were reported by these workers: 35 per cent cream, 66 to 68° C.; 20 per cent cream, 70 to 72° C.; whole milk, 74 to 76° C.; and skimmilk, 76 to 78° C. Josephson and Doan (75) also report that cream has a lower critical temperature than milk.

Proteins associated with fat globules: The lower critical temperature for sulfide liberation from cream observed by Gould and Sommer (45) and Josephson and Doan (75) indicates that the material contributing greatly to the heat labile sulfides of milk products is associated with the fat globules. Gould and Sommer (45) report critical temperatures for 3 minute holding periods of 35 per cent cream and the buttermilk from this cream as 66 to 68° C., respectively, approximately 10° C. lower than that of normal milk. Furthermore, these workers showed that normal 20 per cent cream had a critical temperature of 72° C. for a 3 minute holding period, whereas 20 per cent cream prepared from skimmilk and butter oil had a critical temperature of 79° C., a critical temperature identical to that of skimmilk. In addition, Gould (48) showed that the sulfide contributing material of cream is difficult to remove by washing. Three-times washed cream had a critical temperature practically identical to normal cream.

In contrast, Josephson and Doan (75) were able to remove the sulfhydryl producing materials by washing cream several times and believe that the absorbed layer around the fat globules is a relatively unimportant source of heat-producing sulfhydryl groups. However, they state that this

evidence is not entirely convincing since cream has a lower critical temperature than milk, and has a more sulfide odor and flavor than milk or skimmilk.

Casein: Reasonably convincing evidence has been presented to show that casein as such plays no significant part in labile sulfur formation in heated milk. However, observations on the volatile sulfur of casein have been reported by Osborne (114), Zahnd and Clarke (166), and Blumenthal and Clarke (16). These workers heated casein with basic lead acetate and report sulfur values ranging from 0.07 to 0.10 per cent. Rettger (124) suspended casein in a phosphate buffer (the mixture was still acid to litmus), and upon boiling 100 cc. of a 3 per cent suspension for 40 minutes obtained enough hydrogen sulfide to color lead acetate paper. Ansbacher, Flanigan and Supplee (4) eluted casein with dilute sodium chloride and obtained a protein fraction that appeared to display sulfur migration when exposed to ultra-violet light, heat or agitation. Weckel and Jackson (160) produced an activated flavor in casein by irradiation, and Flake, Jackson, Weckel (36) isolated the activated flavor material from irradiated locust-bean-precipitated casein by steam distillation and adsorption on activated charcoal.

Early studies by Wright (163) and Utz (151) have demonstrated the heat stability of casein toward sulfideliberation. Utz (151) was unable to detect hydrogen sulfide liberation from casein heated to 100° C. in an amphoteric phosphate buffer. Wright (163) used racemisation curves to demonstrate that heat caused no alteration in the constitution of the casein molecule after heating at 60° C. for 1 hour, boiling under reflux for 30 minutes, or autoclaving at 120° C. for 30 minutes. Nickels, Bailey,

Holm, Greenbank and Deysher (110) found that the particle size distribution of calcium caseinate in skimmilk was not affected by momentarily heating at 95° C. Studies by Gould and Sommer (45) and Gould (48) have shown that skimmilk and skimmilk whey have identical critical temperatures, and that buttermilk whey possesses the same critical temperature as buttermilk. Therefore, they concluded that casein of milk contributes few if any of the sulfides liberated at the critical temperature. Similar conclusions were drawn by Josephson and Doan (75).

Whey proteins: Lactalbumin, the principle whey protein has been shown to contain considerable quantities of sulfur, of which approximately 60 per cent is due to cystine (Schmidt (134), Baernsteine (9) and Kassel and Brand (77)). Blumethal and Clark (16) and Zahnd and Clarke (167) have recently reported that allof the sulfur of cystine is heat labile. Mirsky and Anson (106, 107, 108) and Hopkins (64) have demonstrated that -SH and S-S groups appear upon denaturation and coagulation of albumin. Kassel and Branc (77) found that 5 per cent of the sulfur of lactalbumin to be due to cysteine.

Globulin, the other major protein of whey, has been reported by Osborne and Wakeman (116) to contain 0.86 per cent sulfur. Palmer (119) reports a crystalline globulin, which he obtained from albumin, and states that this material appears to contain approximately 4 per cent cystine.

Other sulfur containing materials have been reported as being present in whey and perhaps they too contain heat labile sulfur. Ansbacher, Flanigan, and Supplee (4) isolated the foam producing material from whey and found it to display sulfur migration when subjected to ultraviolet light, heat, or agitation. This material contained no cystine.

Observations on milk have demonstrated rather conclusively that whey proteins are responsible for a great portion of the heat labile sulfur obtained from milk. Gould and Sommer (45) and Gould (48) removed the casein from skimmilk and found that the serum thus obtained liberated hydrogen sulfide when heated to 76 to 78° C. for 3 minutes. The latter also found that the temperature at which sulfhydryls are formed is unchanged when the pH is decreased to 4.6. He states that "albumin may be expected to contribute sulfur compounds on being subjected to heat since it undergoes denaturation and coagulation at the temperatures which produce the -SH groups and hydrogen sulfide, since it has a relatively high sulfur content, and, thirdly since this sulfur is largely supplied by the heat labile amino acid cystine". Josephson and Doan (75) attempted to determine the source of the sulfhydryl compounds formed in heat treated milk and concluded that lactalbumin is their principle origin. Rettger (125) early obtained hydrogen sulfide from lactalbumin even when it was boiled in an acid medium.

Weckel and Jackson (160) were able to obtain an activated flavor in casein-albumin-globulin-free whey separated from irradiated milk. They believe this to be due to absorption. However, Doan and Meyers (28) believe this whey portion of milk to be the source of the "burnt" flavor produced in milk exposed to sunlight.

Since vitamin B₁ contains the thiazole ring as a part of the molecule, there is a possibility that some of the heat labile sulfur of milk is derived from this source. Gortner (44) states that strong alkali and heat causes the liberation of hydrogen sulfide from the thiazole half of thiamin hydrochloride. When milk is subjected to temperatures ranging

from pasteurization to sterilization there may be as much as 10 to 50 per cent of this vitamin destroyed (Daniels, Gibbings and Jorden (24), Holliday (6), Dutcher, Guerrant and McKelvey (29), Krauss, Erb and Washburn (89), and Houston and Thompson (65).

Influence of copper and iron: Small amounts of copper and iron have been shown to retard and in some cases entirely prevent the lability of sulfur. Gould and Sommer (45) found that in normal milk, 76 to 78° C. were sufficient to liberate sulfides, but in the presence of 1.4 ppm. of added copper temperatures of 94° C. or higher are required. Similar results are shown by Gould (48), who found further that the addition of ferrous iron had only a slight retarding influence on sulfide liberation. He also states that copper added to milk after heating is more effective for inhibiting sulfide liberation than when added previous to heating.

Nitroprusside test: The nitroprusside test was employed by Josephson and Doan (75) who found that milk containing 0.5 ppm. of added copper did not give positive tests until heated to 82° C., and that this milk remained positive to nitroprusside for only 4 hours, whereas untreated milk gave positive tests at 76 to 78° C. and remained positive for 48 hours. The test remained positive for as long as 144 hours in milk momentarily heated to temperatures above 80° C.

The nitroprusside test was also used by Gould (48) who reports a positive nitroprusside test on milk containing 1 ppm. of copper when heated to 84° C.; this milk, however, did not evolve detectable hydrogen sulfide until heated at least to 90° C.

Persistency of sulfhydryl compounds: Gould (48) demonstrated that a positive nitroprusside test persists and hydrogen sulfide is evolved throughout a 6-hour aspiration period from milk previously heated momentarily at 90° C., whereas the addition of 1 ppm. of copper immediately after heating and cooling prevented the liberation of hydrogen sulfide and decreased the intensity of the nitroprusside test. Dahle, Lawhorn, and Barnhart (22) report that the addition of 1 ppm. of copper to cream heated to 88° C. momentarily or to 76° C. for 10 minutes resulted in a negative nitroprusside test.

Miscellaneous factors: Additional factors which may influence liberation of sulfides from milk products have been considered. Flake, Jackson and Weckel (36) found that prolonged irradiation of milk caused labile sulfur formation at a temperature approximately 10° C. below normal. They recovered 0.0131 mg. of sulfur from 350 cc. of milk exposed to ultra violet rays for 90 minutes, whereas only 0.0036 mg. were recovered from normal milk. Jackson, Howat and Hoar (68) demonstrated that homogenization had no influence on sulfide liberation in sterilized canned cream.

Certain reagents have been shown to bring about sulfhydryl formation in milk without the application of heat. Jackson (70) and Gould and Sommer (45) obtained positive nitroprusside tests on milk by adding a small amount of sodium cyanide, and Greenstein (54) detected sulfhydryl formation in milk subjected to 8 to 16 molar guanidine hydrochloride. Flake, Jackson and Weckel (36) correlated sulfhydryl compounds with an activated flavor in irradiated milk. In a previous paper (39) they had demonstrated that the flavor could be removed from milk by adding a small amount of hydrogen or calcium peroxide.

Practical Applications of the Formation of Sulfur Compounds by
Heat Treatment

Cooked flavor: Considerable attention is now being given to the problem of cooked flavor in milk and milk products, and recent studies have indicated a relationship between sulfur compounds formed during heating and processing and the production of this flavor (Gould and Sommer (45), Josephson and Doan (75), Gould (48), and Dahle, Lawhorn and Barnhart (22)).

Gould and Sommer (45) present the first positive evidence indicating that sulfur compounds are involved in the formation of a cooked flavor. When milk was subjected to high temperatures the cooked flavor was found to appear rather abruptly with the liberation of hydrogen sulfide at an unusually constant temperature range of from 76 to 78° C. The critical temperature was largely unaffected by such factors as aeration during heating, dilution with as much as 20 per cent of distilled water, separation and remixing of the cream and milk, mastitis infection, seasonal variation, or breed differences. The development of a cooked flavor was enhanced by heating at a pH of 7.6 and retarded by heating at a pH of 5.8 to 6.0. The critical temperature was decreased with a longer period of exposure to heat or by marked increases in fat content.

The addition of 1.4 ppm. of copper prior to heating raised the temperature necessary to produce a cooked flavor to approximately 84° C. When the copper was added following heating, the cooked flavor rapidly disappeared. In other work by Gould (48) he showed that ferrous iron added to milk at the rate of 2.8 ppm. raised the critical temperature only slightly.

The findings of Gould and Sommer (45) were largely substantiated in the studies of Josephson and Doan (75). In addition, the latter workers were able to demonstrate that the appearance of the cooked flavor was simultaneous with the occurrence of sulfhydryl groups as detected by a modified nitroprusside test. This was also shown by Gould (48), who states that this test is more closely correlated with the cooked flavor than the appearance of hydrogen sulfide especially in the presence of added copper.

Several workers have reported on the use of electrical methods of pasteurizing milk as a possible means of using slightly higher temperature than normally employed when other methods of heating are utilized (Prescott (122), Supplee and Jensen (145), and Millenky and Brueckner (103)). In general, it may be concluded from their reports that this method of subjecting milk to heat has no marked influence on the temperature at which cooked flavors and heat-labile sulfur compounds appear.

Activated flavor: Radiation of milk for prolonged periods may cause milk to acquire a flavor defect identified as activated, sunshine or burnt. This flavor is now usually differentiated from an oxidized flavor, but at first was thought to be closely allied. The work of Flake, Jackson and Weckel (36) has shown that ultra-violet rays will liberate sulfhydryl compounds from milk, and have associated these compounds with the activated flavor. Weckel and Jackson (160) produced an activated flavor in casein and albumin by irradiation.

Mancovitz (93) studied ultra-violet rays as a means of pasteurizing milk and states that an exposure of 45 seconds produced

disagreeable sulfur flavors and odors. In addition, Weckel and Jackson (160), Flake, Weckel and Jackson (37), and Flake, Jackson and Weckel (35, 38) demonstrated that a distinguishing characteristic of the activated flavor is that it becomes more noticeable when irradiated milk is momentarily exposed to temperatures above approximately 70° C. These workers found that homogenization rendered milk more susceptible to activated flavor development, and Weckel and Jackson (160) report that milk and buttermilk are more susceptible than cream or skimmilk. The addition of 2 to 3 ppm. of copper causes the activated flavor to disappear.

Doan and Meyers (28) present a rather thorough study showing that sunlight produced the activated flavor in milk products, but made no attempt to correlate their findings with the formation of sulfhydryl compounds. However, Young (164) has demonstrated that sunlight has a destructive effect on albumin, and Ansbacher, Flanigan and Supplee (4) concluded that ultra-violet rays alter the sulfur compounds of the foam producing material of milk.

Oxidized flavor prevention: Oxidized flavor often occurs in processed dairy products especially in the presence of certain heavy metals. The beneficial effect of high temperature pasteurization has been recognized as a preventive measure. A temperature of 85° C. for 5 minutes was employed by Kende (79) to retard and in some cases prevent oxidized flavor development, even in the presence of as much as 2 mg. of CuSO_4 per liter of milk. Kende was of the opinion that an enzyme which he called "oleinase" was catalyzed by metals to develop the oxidized flavor, and that high temperatures inactivated this enzyme. Normal pasteurization temperatures were reported by Dahle and Palmer (23) to enhance the degree of oxidized flavor which may develop. These workers favor the enzyme theory of oxida-

tion since temperatures slightly above pasteurization temperature did not prevent oxidized flavor development, whereas, temperatures above 71° C. for 30 minutes were effective. Ritter (127) emphasizes the beneficial effect of high temperature pasteurization in preventing copper-induced oxidized flavor.

Work dealing with sulfhydryl formation and the oxidized flavor has largely refuted the enzyme theory. Gould and Sommer (45), Josephson and Doan (75), Gould (48), and Swanson and Sommer (147), have shown that high temperature treatment of milk lowers the oxidation-reduction potential, and prevents copper induced oxidized flavor development. This lowering of E_h was correlated with reduced sulfur compounds formed in the milk upon heating. Copper added to milk after heat treatment tends to cause a more frequent and more intense oxidized flavor than when copper contamination occurs before heat treatment, and an oxidized flavor does not develop in milk even in the presence of small amounts of copper until after the sulfhydryl groups have disappeared (48). Gould and Sommer (47) have also shown that milk containing 1.4 ppm. of copper had a higher critical temperature for sulfide liberation than for cooked flavor; this indicates that hydrogen sulfide may combine with the added copper. The importance of sulfhydryl compounds in preventing oxidized flavors in the storage of sweet cream has been demonstrated by Dahle, Lawhorn and Barnhart (22).

The failure of milk to dissolve copper at high temperatures is also involved in the heat prevention of the oxidized flavor. Milk has been shown to practically lose the ability to dissolve copper when heated to high temperatures. (Rice and Miscall (126), Miscall, Cavanaugh and Carodemos (108), and Gebhardt and Sommer (43)). The latter investigators point out that the addition of copper to milk increases the oxidation-

reduction potential but that there is a decided drop in the potential of milk heated above 70° C., temperatures at which the solubility of copper is greatly decreased.

Sulfur Distribution of Milk

Total sulfur: Although different methods have been employed to estimate the sulfur content of milk, the values reported by various investigators agree closely. The percentages reported by several workers are as follows: Sherman (139), 0.034; Tillsman and Sutthoff (149), 0.037; Revol and Paccard (125), 0.027 to 0.044; Steffen and Sullmann (145), 0.030; Kemmerer and Boutwell (78), 0.028; Masters and McCance (96), 0.029; Beach, Bernstein and Hoffman (11), 0.033; and Hutchinson (66), 0.031. Masters (97) has presented an excellent review of the limitations of the methods most applicable to total sulfur determinations of milk products.

The sulfur distribution in milk has been studied by Tillsman and Sutthoff (149) and Beach, Bernstein and Hoffman (10). Tillsman and Sutthoff present the following percentages showing the sulfur distribution of milk containing 92.1 mg. of SO_3 per liter; protein sulfur 84.9; other organic sulfur 4.9; sulfuric acid already formed, 10.4; and ash 1.23. Beach, Bernstein and Hoffman obtained 7.3 mg. of cystine and 86.6 mg. of methionine from the casein present in 100 cc. of milk and obtained 15.5 mg. of cystine and 17.1 mg. of methionine from the whey proteins in 100 cc. of milk. The data of the latter workers show that approximately 80 per cent of the protein sulfur of milk is due to methionine.

Protein sulfur: The three recognized proteins of milk; i.e.,

casein, albumin and globulin, are considered to be good sources of sulfur and have been studied considerably in this regard. A summary of the total sulfur and the portion of the total sulfur contained in the amino acids of the principle milk proteins is reported in Table 1. The total sulfur values reported for casein are consistently near 0.80 per cent. However, the total sulfur values for lactalbumin vary from 1.22 per cent to 1.92 per cent. The cystine and methionine values also show considerable variation.

Table 1. Total sulfur and amino acid sulfur of milk proteins.

Milk Protein	Total Sulfur		Portion of Total Sulfur		Authority
	Per cent	Per Cent	Cystine	Methionine	
Casein	0.80-0.90			84.2-90.2	Baernstein (8)
"	0.80	9.8		83.4	Baernstein (10)
"	0.63				Blumenthal and Clarke (16)
"	0.71-0.78				Zahn and Clarke (166)
"	0.70-0.80	12.0		88.0	Kassell and Brand (78)
"	0.796	8.7		83.7	Beach, Bernstein and Hoffman (11)
Albumin	1.45	62.1		34.4	Baernstein (10)
"	1.22				Blumenthal and Clarke (16)
"	1.92				Osborne and Wakeman (117)
"	1.42	53.0		42.0	Kassell and Brand* (78)
Globulin	0.86				Osborne and Wakeman (117)

* These authors report that 5 per cent of lactalbumin sulfur is due to cystine.

Although Kassell and Brand (77) have been able to account for all of the sulfur in casein and albumin as cystine, cysteine and methionine, other investigators have been unable to find this to be true, especially in the case of casein.

There are relatively few sulfur-containing amino acids, although the list is steadily being increased. Schmidt (134) states that cystine,

cysteine, methionine, and djenkolic acid are the four sulfo-amino acids present in proteins even though indirect evidence points to the possibility of others. Van Veen and Hyman (153) isolated djenkolic acid from the djenkol nut and Horn, Jones and Ringel (64) found a new sulfur-containing amino acid (lanthionine) in sodium carbonate treated wool. Blumenthal and Clarke (15) recently reported that proteins contain at least two sulfur bearing constituents besides cystine and methionine. According to Gulland and Morris (54) acid hydrolyzed casein yields a fraction from which sulfur in elemental form is readily split off.

A summary of the cystine and methionine values of lactalbumin and casein is presented in Table 2.

Table 2. Amino acid distribution in milk proteins.

Milk Protein	: Cystine	: Methionine	: Authority
	: Per Cent	: Per Cent	
Casein	: 3.31-3.10	: Baernstein (9)	
"	: 0.30	: 3.4	: Schmidt (135)
"	: 0.21-0.47		: Vickery and White (157)
"	: 0.34		: Jones and Gersdorff (75)
"	: 0.20-0.30		: Folin and Marenzi (40)
"	: 0.28	: 3.10	: Beach, Bernstein and Hoffman (11)
	: :	: :	
Lactalbumin	: 4.30	: 2.60	: Schmidt (135)
"	: 2.65		: Vickery and White (157)
"		: 2.45-2.32	: Baernstein (9)
	: :	: :	

Considerable variation is evident in the case of the values reported for cystine. Vickery and White (156) believe the variation in cystine values reported for casein is due to methods of purification, and showed that the cystine content of casein decreased each time it was washed with dilute alkali.

Early experiments by Osborne and Wakeman (116) on milk proteins have shown the presence of an alcohol-soluble protein in acid-precipitated casein. In another study (117) these authors found that this alcohol-soluble protein contained 0.95 per cent sulfur, distinctly more than casein.

Several studies have been conducted dealing with the protein compounds in whey freed of casein, albumin and globulin. Osborne and Wakeman (117) indicated that a protein fraction remained after removal of casein, albumin and globulin from milk. Sure and O'Kelly (146) removed these three proteins from milk and report that 66 to 76 per cent of the sulfur remaining was of an organic source. In a recent study by Ansbacher, Flanigan and Supplee (4), a foam producing fraction was isolated from casein-albumin-globulin-free milk. This protein fraction contained 0.60 per cent sulfur, but no cystine. However, Josephson and Doan (75) were unable to obtain a positive nitroprusside test on such whey after heating at 180° C. for 10 minutes.

Several workers have isolated the adsorbed material surrounding the fat globule and have indicated that this material is not identical with other milk proteins. The following percentages of sulfur have been reported for the adsorbed material: Hattori (56), 2.58; Titus, Sommer and Hart (150), 0.74; Palmer and Wiese (122), 0.96; Wiese and Palmer (162), 0.96; and Palmer and Samuelson (120), 0.73. Davies (26) calls attention to a mucoprotein, which was isolated from butter. He states that the adsorbed protein on fat globules has been found to be high in arginine and low in histidine and cystine.

Non-protein sulfur: Surprisingly little convincing information is available regarding the sulfur content of the non-protein fraction of

milk. Early work on this problem by Tillsman and Sutthoff (149) has shown that cows milk contains 15.1 per cent of its sulfur in the form of non-protein organic sulfur. Sure and O'Kelly (146) studied the sulfur distribution of the filtrate obtained from milk after precipitating the casein with acid and precipitating albumin and globulin by heating the acid whey. They report that the dry residue of protein-free milk contains 0.11 to 0.14 per cent sulfur, and that 65.9 to 76.4 per cent of this sulfur is in the organic form.

Viale (154) found that fresh milk reduced methylene blue and gave positive nitroprusside test, concluding from this test that cysteine was present. Martini (94) claimed to have isolated 8 mg. per cent of glutathione from a liter of milk. If present, glutathione may be a source of labile sulfur. Kinsey (81) has demonstrated the destructive effect of x-rays on aqueous solutions of glutathione, and Hopkins (62) removed sulfur from glutathione solutions by gentle aeration of pH 7.6 with a small amount of iron, or by boiling in water. However, Jackson (70), and Gould and Sommer (45) could not obtain positive nitroprusside tests on milk unless a small amount of sodium cyanide was added. Jackson (71, 72) concluded that no cysteine or glutathione are present in free solution in milk, basing this conclusion on the fact that the protein-free filtrate of milk failed to give a positive nitroprusside test when treated with sodium cyanide, whereas a positive test was obtained on the filtrates from milk to which 1 mg. of either reduced glutathione or cystine hydrochloride has been added before removal of the milk proteins. Studies conducted by Gould (46, 47, 49, 50) also indicate that milk does not contain glutathione.

Vitamin B₁ has been shown by Daniel and Munsell (25) to be present in milk to the extent of 20 Sherman units or 30 micrograms per 100 grams of milk; this vitamin contains about 12 per cent sulfur according to Wintersteiner, Williams and Ruehle (162).

Additional Heat Changes in Which Milk Proteins are Involved

The temperature producing hydrogen sulfide, sulfhydryl groups, cooked flavor, and decreased oxidation-reduction potential appears to coincide in a general way with those temperatures causing an increased tendency toward browning, coagulation of the soluble proteins and redistribution of nitrogen.

Discoloration of milk: When milk is subjected to sufficiently high temperatures for a sufficient period of time a distinct browning occurs. Numerous studies have been conducted in order to determine the nature of this discoloration, but as yet this phenomenon has not been fully explained. Webb and Holm (158), Webb (157) and Ramsey, Tracy and Ruehe (123) have pointed out that this discoloration is a function of time as well as temperature. Milk forewarmed at a high temperature for a relatively short time shows greater discoloration than when heated longer at a lower temperature. The latter authors demonstrate that sweetened condensed milk which had been forewarmed in the presence of corn sugar at 88° C. for 15 minutes was only slightly tan in color, whereas the same milk developed a dark brown color when held at 120° C. for 5 minutes.

A protein-sugar complex has been thought by many to be the product responsible for the browning of milk. In this connection, rather comprehensive studies of the reaction between amino acids and reducing sugars have been conducted by Ambler (3), von Euler and Josephson (31),

and Ramsey, Tracey and Ruehe (123). These authors have demonstrated that sugars possessing either aldehyde or ketone groups will condense with the amino group of various amino acids to produce a complex, highly colored substance. The latter authors state that this reaction is more pronounced with lysine and glycine and that sugars having a free aldehyde group, i.e., dextrose, glucose, and levulose, apparently react with free amino groups of the casein. With casein, this reaction may take place at temperatures as low as 40° C., but is enhanced by higher temperatures with milk; the reaction is accompanied by a decrease in pH. Sucrose was found not to form this complex unless it becomes inverted. Kometiani (87) states that caramelization of the lactose is the cause for the browning of milk by heat, and that the reaction is dependent upon the character of the dissolved salts. However, Leeds (9) attributes the formation of brownish-yellow color occurring during the sterilization of milk only partially to the formation of decomposition products of milk sugar since mixtures of casein and albumin removed from discolored milk had a strong yellow color. No off color was apparent in the whey thus obtained. Ramsey, Tracy and Ruehe (123) and Webb (157) state that the complex formed during sterilization is removed with the proteins, and that the color-complex is so firmly attached to the milk proteins that it is not washed away with water, dilute acid, alcohol or ether.

Ramsey, Tracy and Ruehe (123) found that browning could be prevented with a small amount of formaldehyde, citing this as evidence refuting the caramelization theory. They believe that the formaldehyde blocks the amino groups and amino acids of proteins, thus preventing them from condensing with the lactose. As further proof that the off-color of sterilized milk is not due to caramelization of lactose, they ob-

served that solutions of either lactose, casein or albumin failed to change color when held at 250° F. for 30 minutes. Webb (157) used formaldehyde in his experiment and states that "the action of formaldehyde appears to be more or less independent of the amino acid content of the lactose solution". He found that small amounts of formaldehyde markedly increased discoloration, whereas larger amounts inhibited color formation. However, these investigators (123, 157) agree that the browning of sterilized milk is dependent upon free amino groups contained or formed by protein decomposition.

Kass and Palmer (76) completed a recent study on the browning of autoclaved milk and concluded that the development and properties of the brown substance in autoclaved milk are not in harmony with the assumption that it is a casein-lactose condensate. They conclude further that "the exact parallelism between the reactions of caramel, prepared either through the agency of heat alone or heat in the presence of buffer, and those of the brown substance present in heated milk point to the conclusion that the origin and behavior of this coloration may be satisfactorily accounted for on the basis of the caramelization of lactose by the casein and the adsorption of the lacto-caramel by the colloidal caseinate". They present data demonstrating the essential role of casein in the discoloration reaction.

Von Euler and Josephson (31) demonstrated that amino acid-lactose condensation is enhanced with an increasing alkalinity which favors the formation of a free aldehyde group in the sugar molecule. Ramsey, Tracy and Ruehe (123) found that the addition of minute amounts of carbonates or phosphates enhanced color formation in sweetened condensed milk, due to the increase in alkalinity. Webb and Holm (158) found that small amounts of

phosphates, citrates or lime-water failed to produce any change when added to evaporated milk previous to sterilization; however, sodium bicarbonate caused a darkening of color. Further studies by Webb (157) emphasizes that the discoloration during sterilization is of a catalytic nature and is enhanced by phosphates and metals, but prevented by sodium sulfite. Kass and Palmer (76) believe that the dissolved salts of milk have only a negligible influence on heat discoloration.

Heat coagulation of soluble proteins: The soluble protein fraction of milk is often designated as albumin even though it includes albumin and globulin. Rupp (132), Grimmer, Kurtenacker and Berg (53), Matsua (98), Rowland (128, 129, 130), Kieferle and Gloetzi (80), and Irvine and Sproule (67) subjected milk to various temperatures and found that albumin and globulin precipitation does not occur abruptly at any given time and temperature, but begins to precipitate at temperatures above 60° C. and is not completely precipitated until held at 100° C. for 10 to 30 minutes.

Rupp (132) reported that no albumin was coagulated in milk during a 30 minute holding period at 62.8° C. However, 30.78 per cent of the albumin present in milk was precipitated when held for 30 minutes at 71.1° C. Similar observations are reported by Rowland (128) and Irvine and Sproule (67). In other studies (129), Rowland found that maximum precipitation of albumin and globulin occurred when milk was heated to 80° C. for 60 minutes; 90° C. for 30 minutes; 95° C. for 10 to 15 minutes and 100° C. for 5 to 10 minutes. He found that approximately 75.2 per cent of the total soluble nitrogen was albumin and globulin nitrogen.

Studies on the influence of heat on the nitrogen distribution

of milk are reported by Grimmer, Kurtenacker and Berg (53) and Kieferle and Gloetzl (80). A portion of the results reported by the latter workers on the nitrogen distribution of milk heated to various temperatures and held for 30 minutes is presented in Table 3.

Table 3. Influence of heat upon nitrogen distribution in milk.

	: : Raw : :mg. % :	: : 63° C. : :mg. % :	: : 85° C. : :mg. % :	: : 100° C. : :mg. % :	: : 115° C. : :mg. % :
Total N	: 540.4 :	: 537.8 :	: 537.3 :	: 540.5 :	: 537.2 :
Casein	: 348.3 :	: 335.6 :	: 347.8 :	: 383.0 :	: 390.6 :
Albumin	: 75.7 :	: 71.7 :	: 53.3 :	: 13.9 :	: 8.0 :
Proteose	: 44.6 :	: 46.0 :	: 45.7 :	: 42.4 :	: 36.4 :
Peptone	: 45.2 :	: 59.4 :	: 60.0 :	: 66.0 :	: 68.4 :
Total Resi- dual N	: : 123.6 :	: : 138.0 :	: : 148.6 :	: : 152.0 :	: : 160.0 :
	: :	: :	: :	: :	: :

These data indicate that a small portion of albumin is precipitated when milk is held for 30 minutes at 63° C.; approximately one-third of the albumin is precipitated when the milk was held at 85° C. However, the albumin was not completely precipitated even though the milk was held at 115° C. for 30 minutes.

Influence of sugars upon protein coagulation: Comparatively little information is available regarding the influence of sugars upon the heat stability of milk proteins. However, early observations by Leighton and Mudge (92), in a study of the influence of forewarming of milk on the heat stability of the evaporated product during sterilization, have shown that the heat stability of normal milk is increased to a maximum by heating to 95° C. for 10 minutes. However, milk treated with approximately 20 per cent sucrose did not reach maximum heat stability until held for 30 minutes at 95° C. These authors believe that the protective action of sucrose solutions are due to the affinity of sucrose for

calcium and phosphates, which may shift the salt balance to increase the heat stability of the milk.

The possibility exists that sucrose may exert some influence upon the amount of the soluble proteins coagulated during the heating of milk. Beilinson (12), Duddles (30), and Fay (32) have demonstrated that sugars tend to inhibit the heat coagulation of egg albumin at temperatures of approximately 70° C. Duddles determined the coagulation of egg albumin at pH 4.6 when held at 70° C. for 10 minutes, and found that glucose and fructose exerted increased protective action against coagulation with increased concentration. Complete protective action was afforded when the saturation point was reached. Sucrose, mannose and mannitol exerted some protective action, but were less effective than glucose and fructose.

Influence of formaldehyde upon protein coagulation: Evidence is available indicating that formaldehyde influences the coagulation properties of serum proteins. This information, however, has not been directly applied to milk; although, as indicated in the previous section, the action of formaldehyde has been studied in connection with browning.

Early studies of Blum (14) showed that a few drops of formaldehyde prevented the heat coagulation of albumin. Fischer (33, 34) found that 0.0045 to 0.022 per cent of formaldehyde would delay or inhibit the denaturation of serum globulin held at 70° C. This treatment was more effective when the formaldehyde was added soon after denaturation started and had no effect if added 5 or 6 minutes later. However, Freeman (40) demonstrates that serum proteins are 25 to 70 per cent denatured by treatment with 0.5 formaldehyde and holding at 37° C. for 10 days.

Hensley (58, 59), and Marton and Reiner (95) have demonstrated that the addition of formaldehyde caused a gelation of the protein. Hensley believes this effect to be due to condensation of protein molecules. Clark and Shenk (21) believe that formaldehyde reacts at the amide nitrogen groups of protein, tying adjacent chains together. Schiff (133) and Sorenson (143) believe that proteins are denatured by formaldehyde reacting with the free amino group, thereby converting proteins to acids. Sorenson states that formaldehyde reacts with amino acids to form either a methylene derivative or an aldehyde of ammonia.

Influence of metals on the heat coagulation of proteins:

Numerous papers have pointed out that certain metals combine with proteins and amino acids in rather definite proportions to form stable complexes. However, the ratio at which different metals combine with protein has been shown to vary.

Osborne and Leavenworth (115) demonstrated that copper combined with the peptide nitrogen of edestin in the ratio of 1 to 4. Born (16) added copper sulfate and sodium hydroxide to edestin, gelatin and serum protein to show that these substances formed compounds containing from 4.2 to 6.3 per cent copper. Schorn (135) and Bechhold (11) found that zinc, chromium and aluminum combined with albumin at the rate of 1 gram equivalent of metal to 5100-5200 grams of protein. Silver also combines with albumin at this ratio according to Schorn (135) and Heyman and Oppenheimer (60). The latter authors found iron to combine with albumin at approximately twice the above ratio. Northrop and Kunitz (112) found that copper, magnesium, aluminum and silver formed stable compounds with

gelatin. The copper combined at the ratio of 0.9 millimoles per gram of gelatin.

In general, an alkaline medium has been used to bring about the formation of the metal-protein complex. Vickery and Gordon (155) found that mercuric chloride formed complexes most favorably with amino acids at pH 9.3 to 9.8. The complex contains mercury and nitrogen in the ratio of 3 to 2, and also contains chlorine and the base employed for precipitation. Jesserer and Lieben (72, 73) have shown that a number of metals react with casein in the presence of alkali. Kober and Sugiura (86) prepared copper salts of amino acids, peptides and peptones by adding copper hydroxide and holding at low temperatures. Borsook and Thimann (17) studied the influence of hydrogen-ion concentration on the equilibrium relations existing between cupric ions and glycine and alanine at room temperature, and concluded that at least four types of complexes may exist. The copper tended to combine with the carboxyl groups in acid solution and with nitrogen groups in neutral and basic solution.

Metals are attached to the nitrogen of the peptide linkage according to Osborne and Leavenworth (115), Northrup and Kunitz (112) and Jesserer and Lieben (73). However, Smythe and Schmidt (141) noted a correlation between the iron bound casein and the number of free carboxyl and phosphorus groups in the casein molecule.

Michaelis (102) has shown on the basis of oxidation-reduction reactions that cobalt and iron form complexes with cysteine within the pH range of 7 to 8.5. The latter catalyzes the oxidation of cysteine. The amino group plays no part in the reaction, which appears to be due to the simultaneous presence of an -SH and -COOH group in the molecule. Galuialo

and Dobrotworskaja (42) state that proteins increase the oxidative and catalytic action of metals by protecting them in solution.

The influence of metals per se on the heat coagulation of proteins has not been fully established. However, Benedicenti and Rebello-Alves (13) state that metal protein complexes are no longer coagulable by heat. Numerous workers, including Sommer and Hart (142), Webb and Holm (159), Miller and Sommer (104) have shown the salts of certain metals to influence the heat stability of milk proteins. However, this influence concerns the salt balance of the milk rather than the action of specific metals upon the proteins in question.

Influences of alcohol on the heat coagulation of proteins: Although alcohol is used in the precipitation of certain proteins, only limited attention has been given to its influence upon the heat coagulation of these proteins.

Henkel (57) was one of the first to show that an equal volume of 70 per cent alcohol does not always completely coagulate fresh mixed-herd milk. He believes the coagulation action to be due to dehydration of proteins. However, Vasil'ev (152) measured the coagulating action of various alcohols on albumin and casein and found that coagulation begins at such low concentration of alcohol that the action is not one of dehydration, but is a surface energy change resulting from the adsorption of alcohol on the protein micelle.

Klobusitzky (85) and Teorell (148) have shown that even small amounts of alcohol lower the temperature of protein coagulation. Klobusitzky (85) found that pseudoglobulin manifests its greatest turbidity below the boiling temperature with 18 per cent alcohol. Teorell (148) shows that

protein coagulum formed in such concentrations of alcohol tend to dissolve when heated to boiling and reprecipitate on cooling.

Harris and Matill (55) found that extraction of kidney and liver globulins with boiling 95 per cent alcohol produced no decrease of nitrogen in these ptoreins, but both of the proteins show a loss of cystine and hydrogen sulfide. Hopkins (65) states the precipitation of protein from solution with alcohol causes the appearance of -SH groups.

SCOPE OF INVESTIGATION

This study was conducted to obtain information concerning the volatile sulfides of milk products and the influence of certain factors thereon. Data were secured with three main objectives in mind:

(a) To determine in a general way the origin of the heat labile sulfur compounds of milk by means of a quantitative method, and to determine the relationship of volatile sulfide liberation to total sulfur, total nitrogen and albumin nitrogen.

(b) To determine the influence of various heat treatments upon the heat labile sulfur compounds, cooked flavor and color changes of milk.

(c) To determine the influence upon the heat volatile sulfides of the addition of (1) sugars, (2) ethyl alcohol, (3) sodium chloride, (4) cystine or cysteine, (5) reducing substances, (6) hydrogen peroxide, (7) acids or alkali and (8) metals.

EXPERIMENTAL PROCEDURE

General methods: Milk used in this study was obtained either by random selection from the daily deliveries received by the College Creamery, or, in case of controlled experiments, directly from the college herd. Such milk was used immediately either directly in experimental studies, or for preparing other milk products. Cream and skimmilk were secured by mechanical separation of the fresh milk; buttermilk by churning the fresh cream; and whey and buttermilk whey by rennin coagulation of the casein in the skimmilk or buttermilk. Samples used in homogenization studies were momentarily heated to 135° C. to inactivate lipase (Gould (51)). All samples were secured and handled in well-tinned or glass containers to avoid copper and iron contamination.

Heating of the samples to temperatures below boiling was accomplished in a water bath. Unless otherwise stated the samples were placed in a round bottom flask and agitated with a glass-rod stirrer, or by aspiration with nitrogen or air. Approximately 15 minutes were required to bring the samples to the desired temperature. The temperature was controlled within an accuracy of $\pm 0.5^{\circ}$ C. Cooling was accomplished by replacing the water in the water bath with cold water, thus bringing the sample to room temperature within 5 minutes.

When samples for flavor or nitroprusside determinations were required, the heating apparatus included a glass-tube siphon which permitted the removal of samples at desired temperatures. Flavor observations were made by two reliable judges, after first adjusting the samples to

approximately room temperature.

High temperature heat treatment of the milk, i.e., temperatures above boiling was obtained by means of a steam autoclave. The milk was usually sealed and heated in No. 2 Canco, plain sanitary cans (American Can Company). Approximately 15 minutes were required to reach the desired pressure. Following heating, the samples were cooled by immediately transferring the cans to cold running water.

Qualitative methods: The qualitative studies on sulfide liberation were conducted according to the procedure adopted by Gould and Sommer (45). The volatile sulfides were recovered on basic acetate paper by bubbling air through 4.5 liter samples while heating to the desired temperature in a 5-liter round bottom flask. The discoloration of the acetate paper indicated the liberation of sulfides. The basic acetate paper was prepared by the method described by Gould and Sommer (45).

Sulfhydryl determinations were made as suggested by Gould (48). A 5 cc. sample was saturated with ammonium sulfate, 10 drops of 5 per cent sodium nitroprusside and 5 drops of concentrated ammonium hydroxide were added. The tube was inverted several times after adding each reagent. The intensity of the pink color appearing indicated the extent of sulfhydryl formation.

Quantitative methods: After conducting a number of preliminary trials involving the use of colorimetric and titration methods for the quantitative determination of volatile sulfides the Sheppard and Hudson (38) modification of the Almy (2) method with some additional variations was adopted. Kirklenko (82) has shown this method to give an accuracy of 0.001

per cent when used to determine the sulfides of geletin. The method involves the collection of the liberated sulfides in alkaline zinc acetate and then the production of methylene blue by the addition of p-amino dimethyl aniline and ferric chloride. The intensity of the blue color varies directly with the amount of sulfides.

Alkaline zinc acetate was prepared by adding 25 cc. of a 20 per cent stock solution of zinc acetate and 40 cc. of 10 per cent sodium hydroxide to a liter flask and making up to volume with distilled water. The indicator solutions consisted of a 0.1 per cent p-amino dimethyl aniline (Eastman Kodak, No. 1333), in 1:1 hydrochloric acid and 0.02 molar ferric chloride (hexahydrate), in 4 per cent hydrochloric acid. The apparatus used for the quantitative determination of volatile sulfides is diagrammatically shown in Fig. 1. The quantitative procedure involved the heating of a 2-liter sample in a 3-liter flask suspended in a water bath. Moderate agitation was produced and the evolved gases were carried off by passing nitrogen gas through the samples during the heating period and for 30 minutes thereafter.

Before passing through the sample, the nitrogen gas was freed from oxidants by bubbling through alkaline pyrogallol and distilled water. The rate of nitrogen flow through the sample was regulated so as to keep 5 bubbles apparent on the surface of the distilled water. The alkaline pyrogallol was prepared as recommended by the chemist of the U. S. Steel Corporation (101), whereby 300 gm. of pyrogallic acid are dissolved in a liter of distilled water and 2.5 volumes of 50 per cent sodium hydroxide added. Rubber stoppers used in the apparatus were freed of sulfur by boiling in strong sodium hydroxide and rinsing in an abundance of distilled

water. Sulfur-free rubber tubing was used for connecting glass tubing. Blank determinations with distilled water showed the apparatus to be free of sulfur.

In order to recover the volatile sulfides, the nitrogen gas was conveyed to the two receivers by a 3-foot condenser and glass tubing. An empty flask was connected in the line between the condenser and the receivers to serve as a trap. The receivers were 50 cm. in height and 20 mm. in diameter. Each receiver was half filled with 4 mm. glass beads and contained 75 cc. of the dilute alkaline zinc acetate. Preliminary trials revealed that practically all of the sulfides were removed in the first receiver.

At the end of the aspiration period, the solution and glass beads were removed from the receivers and the receivers were rinsed with distilled water and then with dilute hydrochloric acid, the rinsings were collected in separate containers. The acid rinse was withheld from the alkaline receiving solution to avoid the possibility of an error due to the liberation of hydrogen sulfide before the indicators were added. The rinsings were then added to the alkaline zinc mixture, along with 25 cc. of the diamine reagent and 5 cc. of ferric chloride and the flask was immediately stoppered. A few minutes were allowed for color development, after which the blue solution was placed into a 300 cc. volumetric flask. The beads and flask were rinsed several times with distilled water, the rinsings were added to the volumetric flask and the flask made up to volume with distilled water. After standing over night the intensity of the blue color was determined with an Evelyn photoelectric colorimeter, using a 620

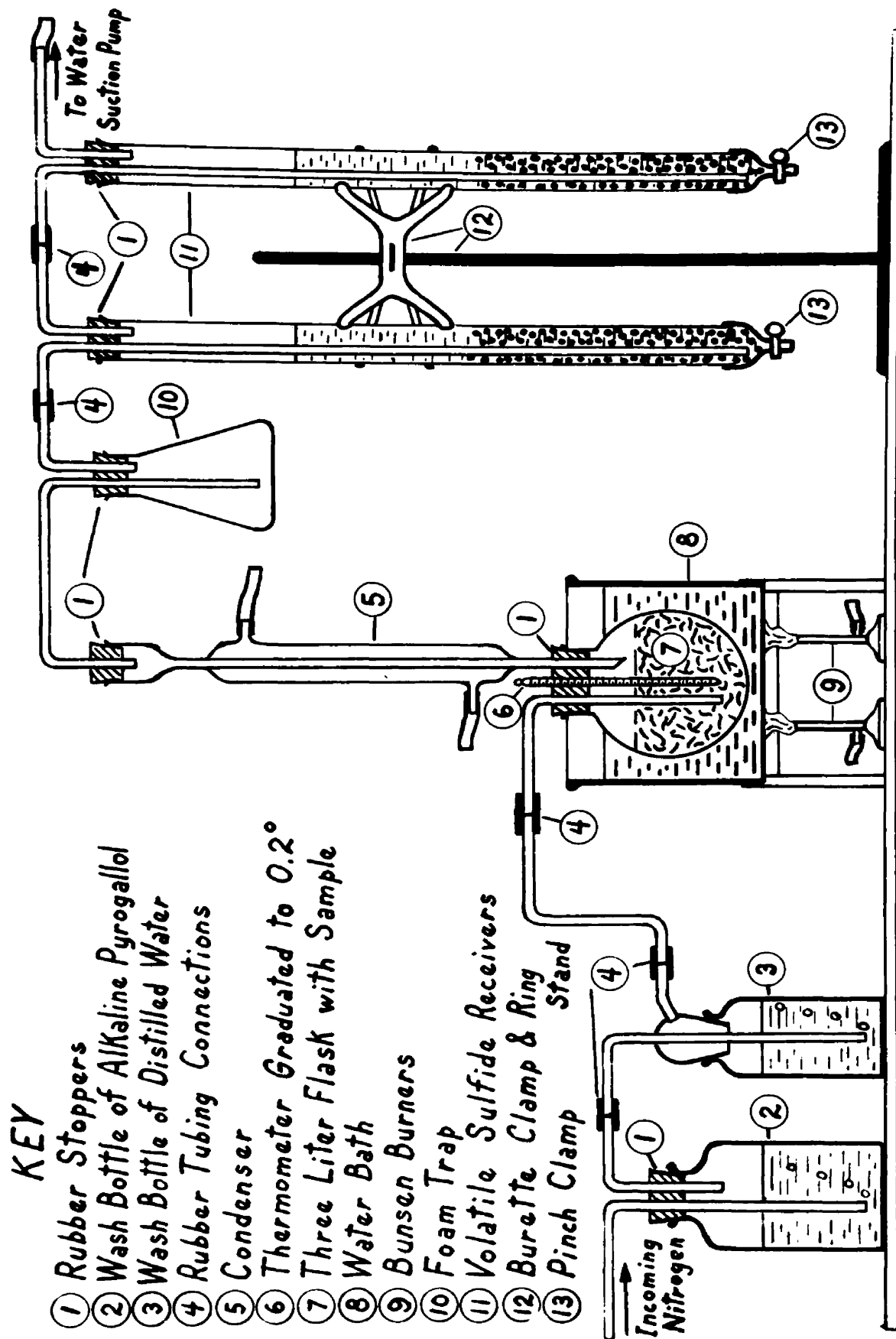


Fig. 1 - Diagrammatic sketch of the apparatus used for the quantitative determination of volatile sulfides.

filter. The photometer readings were referred to a previously prepared standard curve for the sulfur values.

A filter showing maximum light transmission at a wave length of 620 millimicrons was selected after finding that the representative color standard exhibited maximum absorption at a wave length between 600 and 700 millimicrons when observed with a Cenco-Sheard Spectrophotometer. The typical transmission curve is shown in Fig. 2.

The known color standards were obtained by a variation of the Lachele (90) procedure. Accurate amounts of standard sample 14 c Bureau of Standards basic open-hearth steel were placed in the sulfide apparatus and 50 cc. of 1:1 hydrochloric acid were added, the acid alone gave a perfect blank. The sample was gently heated until dissolved and then boiled for 5 minutes. Nitrogen gas was passed through the system during the heating period and for 10 minutes after heating. The hydrogen sulfide thus produced was collected in the receivers and the blue color was developed as described above. The photometer readings produced by recovering the hydrogen sulfide from different weights of steel of known sulfur content are shown in Table 4.

After obtaining the photometer readings of the color standards produced from steel samples containing amounts of sulfur ranging from 0.03 to 1.50 mgs., the standard curve was prepared by plotting the logarithm of the photometer reading of the known standards against their sulfur content. The standard curve is presented in Fig. 3.

Total sulfur was determined on a 25 gram sample according to the official magnesium nitrate method (6). This method was found to be accurate when checked against cystine samples of sufficient size to give barium sulfate precipitates equal to those generally obtained for normal milk. Total and albumin nitrogen was determined on a 5 gm. sample by the

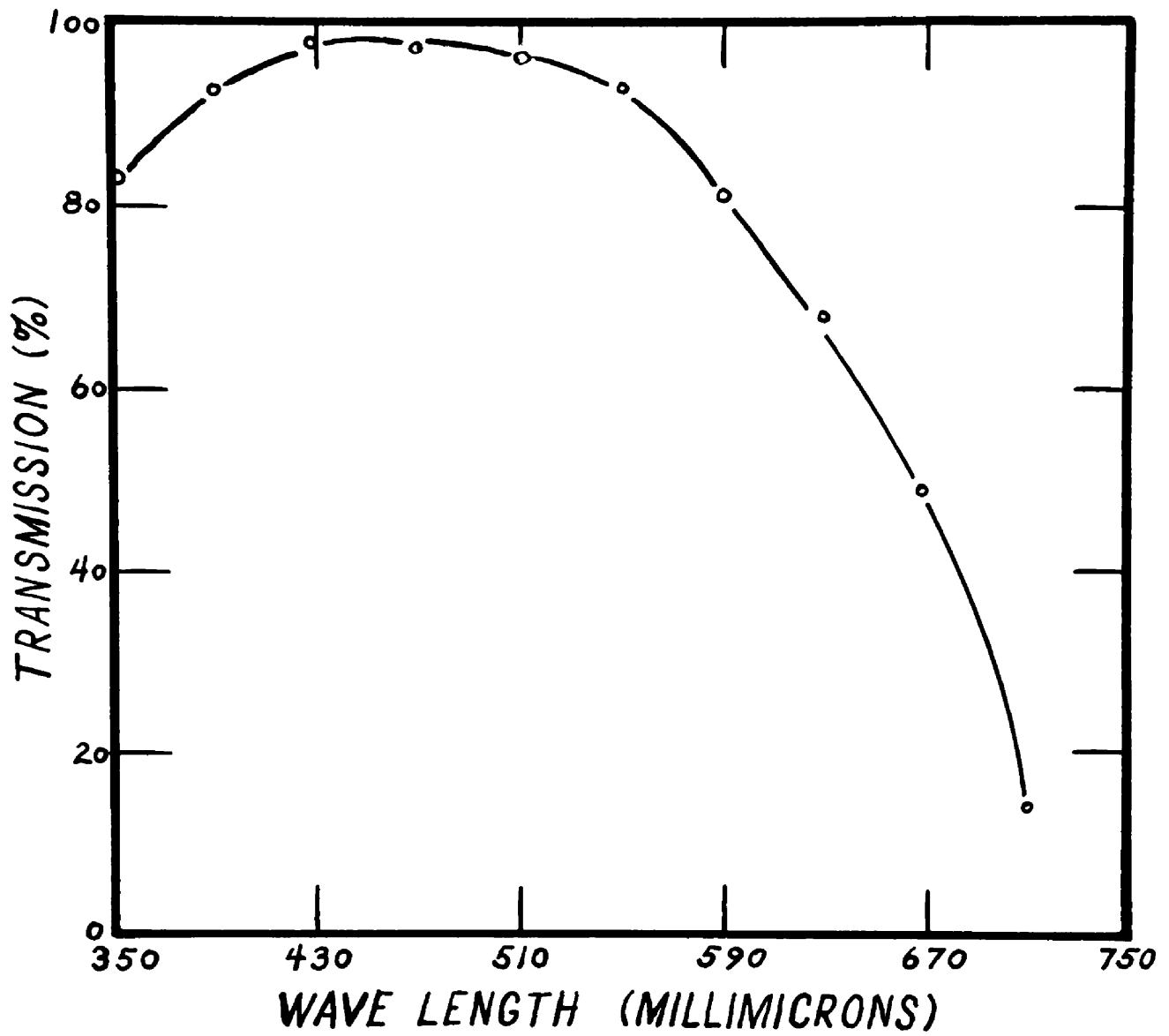


Fig. 2. Typical transmission curve of a representative color standard of known sulfur content.

official method (6).

Table 4. Photometer readings for standard methylene blue solutions prepared from steel samples of known sulfur content*.

Grams of steel used (0.03 % sulfur)	: Milligrams of sulfur in the steel sample	: Photometer readings using 1 cc. of the standard solution and a 620 filter
0.1	0.030	97.0
0.2	0.060	95.5
0.5	0.150	90.5
1.0	0.300	81.0
1.5	0.450	72.0
2.0	0.600	62.0
2.5	0.750	52.0
3.0	0.900	47.5
3.5	1.050	35.0
4.0	1.200	27.0
4.5	1.350	20.0
5.0	1.500	12.7

* Each sample was determined in triplicate.

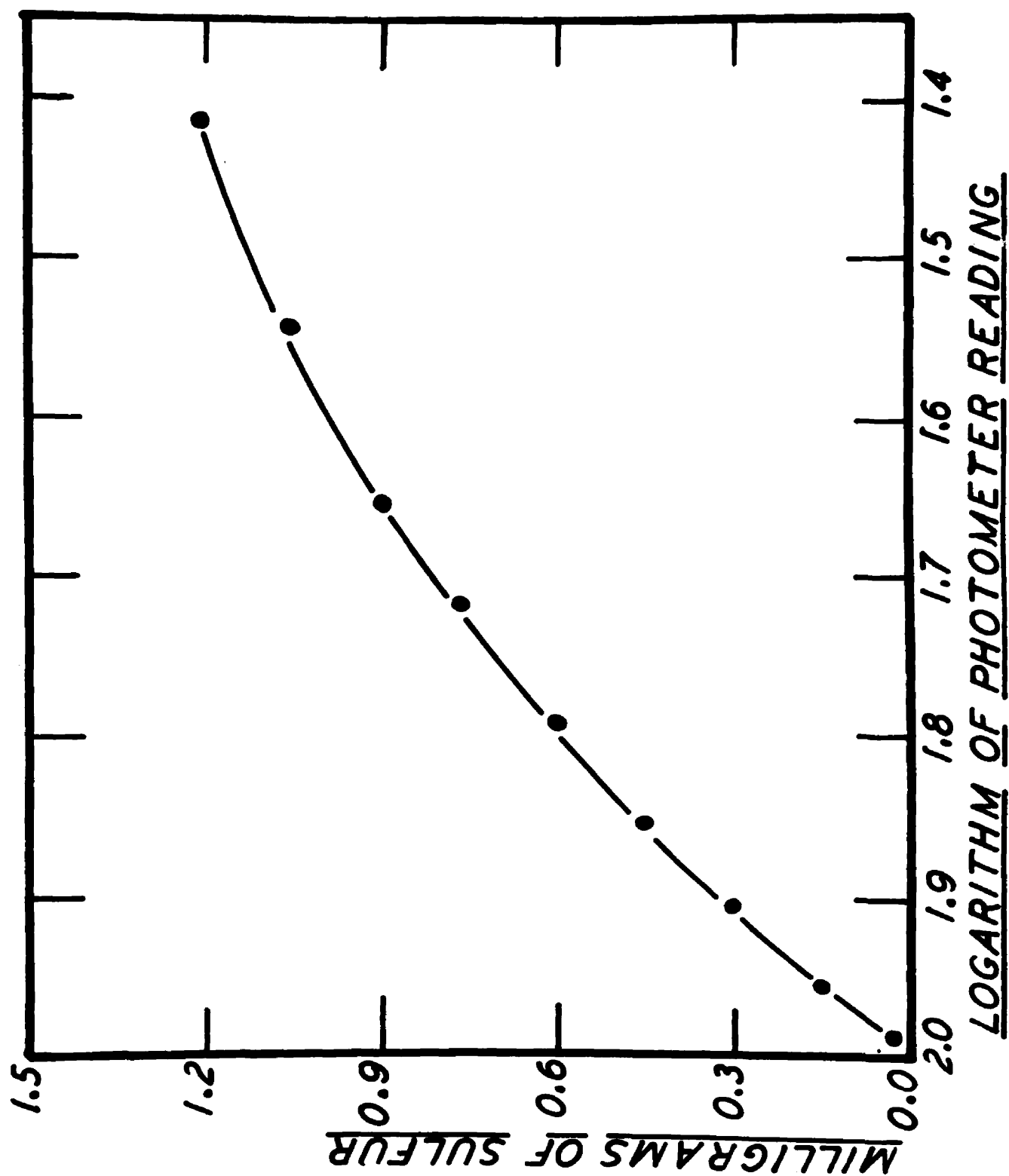


Fig. 3. Standard curve from prepared solutions of known sulfur content.

RESULTS

General Origin and Quantitative Studies of Heat Labile Sulfides

Critical temperature studies: To study quantitatively the critical temperatures of milk, skimmilk and rennet whey, samples were heated momentarily to temperatures ranging from 72° C. to 90° C. The heat labile sulfides evolved during heating and for 30 minutes after cooling are shown in Table 5.

Table 5. Volatile sulfur recovered from milk, skimmilk and whey momentarily heated at different temperatures.

Temperature	Milk**	Skimmilk*	Whey*
°C	(mg/l)	(mg/l)	(mg/l)
72	0.000	0.000	0.000
74	0.000	0.000	0.000
76	0.014	0.000	0.000
78	0.025	0.000	0.000
80	0.053	0.012	0.020
82	0.076	0.026	0.040
84	0.100	0.045	0.065
86	0.130	0.054	0.124
88	0.202	0.065	0.158
90	0.247	0.158	0.205

** Average of 6 determinations.

* Average of 3 determinations.

These data show that a small but measurable amount of sulfides are liberated when milk is heated momentarily to 76 to 78° C. and that the amount of sulfides liberated increase with increases in heat exposure throughout the entire heating range. No sulfides were detected in milk heated to 72 and 74° C., but 0.014 mg. per liter are evolved at 76° C. and

0.247 mg. per liter are liberated at a temperature of 90° C. Skimmilk and whey have critical temperatures from 2 to 4° higher than whole milk, and these two products do not evolve as much sulfide as whole milk. However, these products show the same relationship as whole milk in regard to increased sulfide liberation with increased heat exposure. The skimmilk evolved 0.012 mg. of sulfur as volatile sulfides when heated to 80° C. and 0.158 mg. at 90° C. Whey evolved slightly more sulfides than skimmilk, but less than whole milk, the whey liberating 0.020 mg. of sulfur at 80° C. and 0.205 mg. at 90° C.

The data in Table 5 also show that casein per se is not a contributor of heat labile sulfur. In fact, casein appears to slightly inhibit sulfide liberation as indicated by the difference in values obtained for skimmilk and whey. However, serum proteins, or those proteins in whey, apparently are a definite source of heat labile sulfur. The fact that milk has a lower critical temperature and liberates sulfides in greater quantities than skimmilk and whey indicates a correlation with the butterfat content.

To determine the influence of butterfat and the membrane adsorbed on the fat globules upon the critical temperature and quantity of sulfides evolved, milk, cream (both 20 and 30 per cent), buttermilk and buttermilk whey (rennet) were secured from the same source. Heating trials were then conducted and the amount of volatile sulfide evolved at different temperatures from each of these products are presented in Table 6.

These results show that cream, buttermilk and buttermilk whey have a lower critical temperature than milk. Furthermore, by comparing the data in Tables 5 and 6, it is evident that cream, buttermilk and buttermilk

whey also have lower critical temperatures than skimmilk or whey. These results demonstrate that 30 per cent cream has a slightly lower critical temperature than 20 per cent cream; 30 per cent cream liberated the first detectable sulfides at a temperature of 66° C., whereas 20 per cent cream showed the first measurable liberation of volatile sulfides when momentarily heated to 70° C. In like manner, the corresponding buttermilk and buttermilk whey from 30 per cent cream was found to have lower critical temperatures than the buttermilk and buttermilk whey from 20 per cent cream. The buttermilk has a slightly lower critical temperature than its corresponding cream, and the buttermilk whey has a critical temperature approximately 2° C. below the buttermilk from which it came.

Table 6. Volatile sulfur recovered from milk and its corresponding cream, buttermilk, and buttermilk whey momentarily heated at different temperatures*.

Temperature:	Milk	20% fat	Buttermilk	whey	30% fat	Buttermilk	whey
°C	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)
62	-	0.000	0.000	0.000	0.000	0.000	0.000
64	-	0.000	0.000	0.000	0.000	0.000	0.012
66	0.000	0.000	0.000	0.006	0.000	0.006	0.026
68	0.000	0.000	0.012	0.022	0.006	0.012	0.049
70	0.000	0.006	0.028	0.036	0.010	0.036	0.071
72	0.000	0.012	0.040	0.057	0.019	0.067	0.106
74	0.000	0.028	0.060	0.082	0.045	0.114	0.151
76	0.012	0.033	0.082	0.106	0.067	0.135	0.192
78	0.031	0.095	0.098	0.127	0.151	0.176	0.245
80	0.052	0.151	0.158	0.167	0.252	0.275	0.305
82	0.065	0.196	0.192	0.200	0.345	0.350	0.360
84	0.090	0.237	0.237	0.252	0.400	0.405	0.412
86	0.124	0.260	0.282	0.290	0.430	0.435	0.445
88	0.196	0.275	0.305	0.337	0.462	0.487	0.525
90	0.243	0.296	0.330	0.375	0.480	0.512	0.575

* Average of two trials.

Another point of interest revealed by the data in Table 6 is that cream, buttermilk, and buttermilk whey liberate considerably more volatile sulfides than either milk, skimmilk, or whey. For example, when milk, cream, buttermilk, and buttermilk whey are momentarily heated at 90° C. the following amounts of sulfur per liter were obtained by collecting the volatile sulfides from these products: milk, 0.243 mg.; 20 per cent cream, 0.296 mg.; the buttermilk from 20 per cent cream, 0.330 mg.; and the buttermilk whey from 20 per cent cream, 0.375 mg. The values obtained for 30 per cent cream and its corresponding buttermilk and buttermilk whey are 0.480, 0.512 and 0.575, respectively.

The above values further substantiate the fact that casein apparently does not contribute to the amount of sulfides evolved. This is best observed in Fig. 4 by comparing the similarity in results obtained for buttermilk and buttermilk whey. In fact, buttermilk whey evolves sulfides at a lower temperature and in greater quantities than the buttermilk. In addition this portrayal clearly demonstrates the significance the material adsorbed on butterfat as a major source of heat labile sulfides.

The proteins associated with the fat appears to be the greatest contributor of heat labile sulfides of any of these fractions studied. In Table 6, the results show that as the fat content increases the amount of sulfur recovered as volatile sulfides is also increased. These results show further that buttermilk and buttermilk whey, which contain the fat associated proteins in greater amounts per unit volume than the cream, liberate heat labile sulfur in much greater quantities than an equal volume of cream. The fact that 30 per cent cream and its corresponding buttermilk and buttermilk whey evolve sulfides at lower temperatures and in quantities

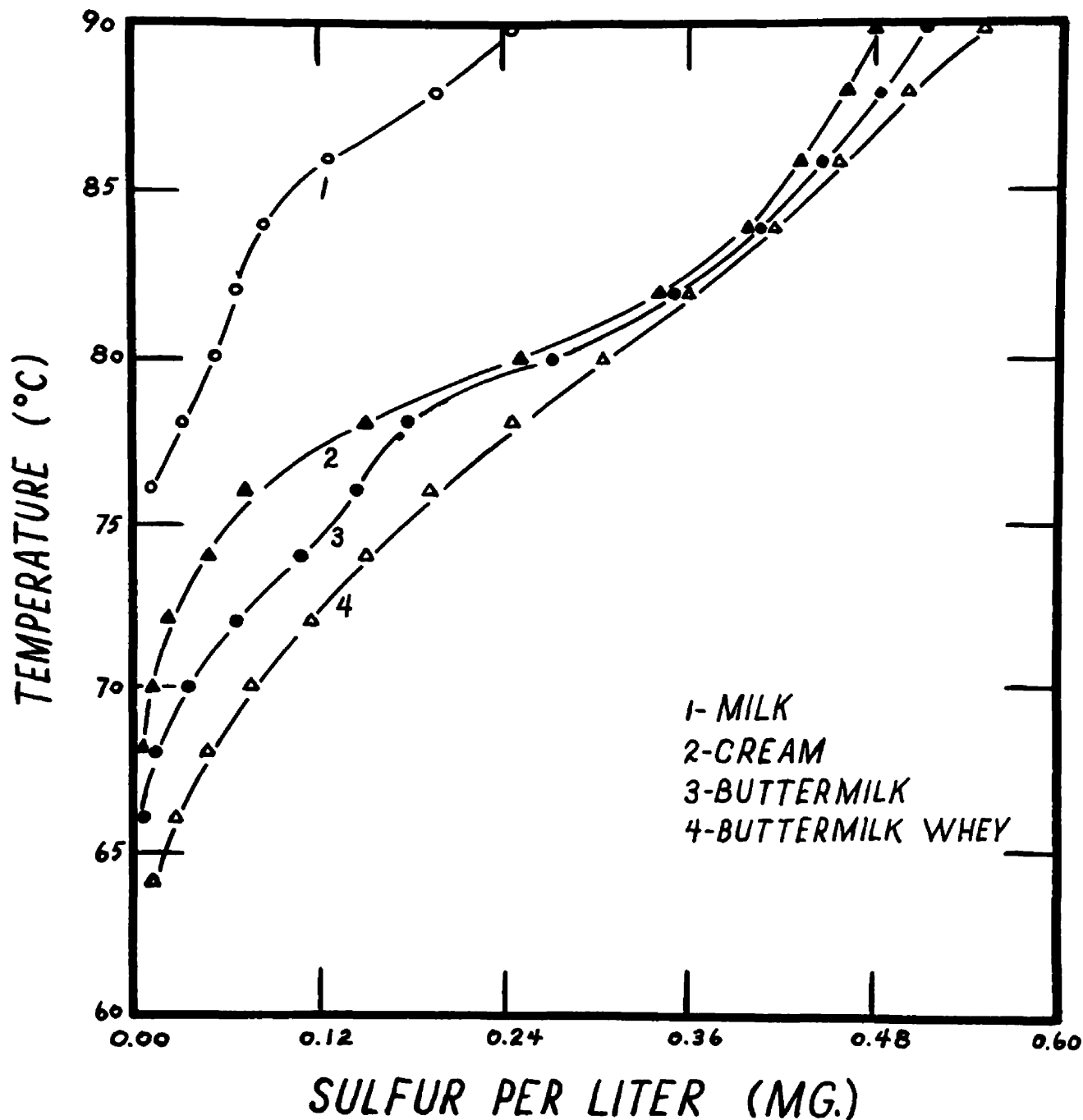


Fig. 4. The critical temperatures of milk and its corresponding cream (30 %), buttermilk and buttermilk whey, and the relationship of the temperature of heating to the quantity of sulfur evolved as sulfides by these products.

approximately twice as great as the milk from which it came is significant proof of the importance of the fat membrane, or at least the fat associated proteins, as a major source of sulfides.

Relationship of heat labile sulfur to total sulfur and total

nitrogen: In order to ascertain more about the origin of heat labile sulfides, and, also, to study the nature of the constituents evolving sulfides when milk products are exposed to heat, various milk products were secured as in the previous section and the volatile sulfides, total sulfur and total nitrogen were determined. These results are presented in Table 7, which presents an average of four typical determinations.

Table 7. Relationship of the volatile sulfides obtained by heating milk momentarily at 90° C. to the total sulfur and total nitrogen*.

Product	:	:	:	:	Ratio	
	:Sulfur recovered	:Total	:Nitrogen:	:	:Volatile sulfur	:Total sulfur
	:as volatile	:sulfur:	:	:	:to	:to
	:sulfide	:	:	:	:total sulfur	:total nitrogen
	(mg/l)	(mg/l)	(gms/l)	:		
Milk	0.227	291	5.62	:	1:1074	1:19.31
Skimmilk	0.175	304	5.89	:	1:1737	1:19.37
Whey	0.192	189	1.76	:	1:984	1: 9.31
Cream**	0.476	235	3.73	:	1:494	1:15.87
Buttermilk	0.523	303	5.66	:	1:579	1:18.68
Buttermilk whey	0.521	220	2.12	:	1:422	1: 9.64
	:	:	:	:	:	:

* Average of 4 determinations.

** Between 30 and 35 per cent fat.

The volatile sulfide values were obtained by aspirating the sample while heating to 90° C., and for 30 minutes thereafter. The total sulfur was determined on a 25 gram sample and the total nitrogen was determined on a 5 gram sample of the original product.

The results on the volatile sulfide determinations compare

the buttermilk whey, this fraction appears to be the factor responsible for the fact that the ratio of volatile sulfur to total sulfur of buttermilk whey is only twice that of whey. However, the ratio of total sulfur to total nitrogen is approximately the same for these two products. These findings indicate that the membrane constituents contain a fraction that is not especially high in sulfur, but is relatively unstable toward heat.

Washing of cream: Trials were also conducted to determine the influence of washing upon the fat globule membrane. In this study, cream of approximately 30 per cent fat was washed three successive times at 37.5° C. with equal portions of distilled water of the same temperature, after each washing and separation the cream was adjusted to the original volume and original fat test with the liquid obtained by the corresponding separation. The results for volatile sulfur at 90° C., total sulfur, total nitrogen and albumin nitrogen are presented in Table 8.

Table 8. Influence of washing of cream on the volatile sulfides, total sulfur, total nitrogen, and albumin nitrogen of the cream and its buttermilk*.

Product	:Sulfur recovered:	Total	Total	Albumin	Ratio	
	as volatile sul-:fides	sulfur	nitrogen	nitrogen	Total sulfur to total nitrogen	Volatile sulfur to albumin nitrogen
	(mg/l)	(mg/l)	(gms/l)	(gms/l)		
Cream**	0.450	212	4.610	0.602	1:21.74	1:1337
Buttermilk	0.489	290	5.680	0.650	1:19.59	1:1329
Washed cream	0.368	95	0.640	0.072	1: 6.74	1:196
Washed cream buttermilk	0.470	104	0.805	0.083	1: 7.74	1:177

* Average of 3 trials.

** Fat content of 25 to 30 per cent.

These results show that the constituent of the fat membrane contributing heat labile sulfur is rather firmly attached. The washed cream has only a slightly lower volatile sulfur value than the original cream, although the values for total sulfur, total nitrogen and albumin nitrogen of the washed cream are considerably lower than those of the original cream. The washed cream buttermilk is also shown to be high in heat labile sulfur and low in total sulfur, total nitrogen and albumin nitrogen. Also, the washed cream and washed cream buttermilk are shown to agree closely in the ratio of total sulfur to total nitrogen and in the ratio of volatile sulfur to albumin nitrogen.

Separation temperature: In a further study of the heat labile properties of the material adsorbed on fat globules, samples of milk were separated at 15.5° C., 35° C. and 57.2° C. In addition, a sample of cream obtained by separating milk at 15.5° C. was re-separated at 57.2° C. to a higher fat content, and then restandardized with the original skim milk, which was secured by separating milk at 15.5° C. Such a study appeared desirable since Sharp (138) has pointed out that the globule membrane apparently contains an adhesive material that remains with the cream when milk is separated at low temperatures, this material passing into the skim milk when milk is separated at high temperatures. The volatile sulfur liberated at 90° C. and the total sulfur were determined on the cream and skim milk secured by separating at each of the above temperatures. These results are presented in Table 9.

The values obtained for the volatile sulfur and total sulfur of the cream or skim milk obtained by separating milk at a low temperature

vary only slightly from those obtained for cream and skimmilk secured by high temperature separation. These data indicate that the adhesive material remaining in cream at low temperature separation and passing into the skimmilk at high temperature separation is either not a major source of heat labile sulfides and is not especially high in sulfur content, or the methods used were not sufficiently sensitive to detect a difference in values.

Table 9. Influence of separation temperature upon the volatile sulfides and total sulfur of the resulting cream and skimmilk*.

Temperature of separation °C	Cream (25 per cent fat)		Skimmilk	
	Volatile sulfur (mg/l)	Total sulfur (mg/l)	Volatile sulfur (mg/l)	Total sulfur (mg/l)
15.5	0.428	245.5	0.156	324.1
35.0	0.397	263.2	0.156	318.6
57.2**	0.408	247.0	0.175	320.0
57.2	0.388	254.0	0.172	318.1

* Average of 2 determinations.

** This was a sample of 25 per cent cream obtained by separating milk at 15.5° C. This 25 per cent was then re-separated to a higher fat content after heating to 57.2° C., the cream sample was then restandardized to 25 per cent butterfat with skimmilk obtained by low temperature separation.

Homogenization: To determine the influence of homogenization on the critical temperature, milk was heated to approximately 57° C. to largely inactivate the lipase (51), and a portion was homogenized at 2500 pounds pressure. The homogenized and unhomogenized milk was then studied from the standpoint of sulfide liberation, nitroprusside reaction and cooked flavor after momentarily heating to temperatures ranging from 70 to 90° C. Results are presented in Table 10.

Table 10. Influence of homogenization upon the sulfide liberation, nitroprusside test and cooked flavor of milk momentarily heated to different temperatures*.

Temperature:	Homogenized*			Unhomogenized		
	Volatile:	Nitroprusside:	Cooked:	Volatile:	Nitroprusside:	Cooked:
	sulfur	test	flavor:	sulfur	test	flavor
	(mg/l)			(mg/l)		
70	: 0.000	: 0	: 0	: 0.000	: 0	: 0
75	: 0.010	: 0	: 0	: 0.015	: 0	: ?
80	: 0.043	: 1	: 1	: 0.052	: 1	: 1
85	: 0.129	: 2	: 2	: 0.129	: 2	: 2
90	: 0.235	: 4	: 4	: 0.237	: 4	: 4
	:	:	:	:	:	:

* Average of two trials. The intensity of the nitroprusside test and cooked flavor is indicated as follows: negative, 0; questionable, ?; slight, 1; definite, 2; pronounced, 3; and strong, 4.

These results show that homogenization of milk has no significant influence on the critical temperature nor upon the amount of heat labile sulfur evolved at the various temperatures. Furthermore, the sulfhydryl reaction as detected by nitroprusside and the cooked flavor of the homogenized and unhomogenized milk are also shown to be practically identical.

Breed and period of lactation: The previous studies were conducted on mixed herd milk. It seemed advisable to demonstrate the influence of the breed of cows and the period of lactation upon the critical temperature, amount of volatile sulfides evolved by heating, and the total sulfur.

For this study four cows were selected, two Holsteins and two Guernseys. Numbers 280 and 48 were just entering their lactation periods and numbers 271 and 51 had been producing for approximately 5 months. This scheme of selecting the animals was followed in order to study the milk from one animal of each breed throughout the entire lactation period.

and also to study the milk from one animal of each breed as she was completing her lactation period and again at the beginning of her lactation period. Unfortunately, number 51 had to be slaughtered during the second month of her new lactation period and only one sample was obtained after she freshened. The milk from these animals was collected over a 24-hour period once each month and the volatile sulfides and also the total sulfur values were determined. In each case the determination was conducted in duplicate and the average results are presented in Table 11.

Table 11. Influence of breed and period of lactation upon the volatile sulfides and upon the total sulfur*.

	Holstein				Guernsey			
	280		271		51		48	
	: Volatile:	: Total	: Volatile:	: Total	: Volatile:	: Total	: Volatile:	: Total
	: sulfur	: sulfur	: sulfur	: sulfur	: sulfur	: sulfur	: sulfur	: sulfur
	: mg/l	: mg/l	: mg/l	: mg/l	: mg/l	: mg/l	: mg/l	: mg/l
July	: 0.208	: 208	: 0.201	: 273	: 0.222	: 270	: 0.223	: 277
August	: 0.218	: 210	: 0.238	: 277	: 0.287	: 273	: 0.228	: 275
September	: 0.245	: 210	: ****	: ****	: 0.286	: 273	: 0.253	: 285
October	: 0.252	: 242	: ****	: ****	: 0.282	: 281	: 0.245	: 279
November	: 0.256	: 247	: 0.192	: 249	: ****	: ****	: 0.234	: 306
December	: 0.250	: 255	: 0.215	: 242	: 0.275	: 278	: 0.267	: 269
January	: 0.245	: 244	: 0.238	: 241	: -----	: -----	: ****	: ****
February	: 0.252	: 252	: 0.238	: 243	: -----	: -----	: ****	: ****
	: :	: :	: :	: :	: :	: :	: :	: :

*These data are an average of duplicate determinations.

**** Dry period.

The volatile sulfur and total sulfur values are shown to increase as the lactation period progresses although some variations occur. The results show that the two samples of Holstein milk agree rather closely for both the total sulfur and volatile sulfur. Likewise, the two samples of Guernsey milk correspond fairly well for both the volatile and total sulfur. However, the Guernsey milk which was slightly higher in fat and thus also in

solids-not-fat, is slightly higher in volatile and total sulfur. Furthermore, in each of the samples of milk there appears to be a rather constant ratio of volatile sulfur to total sulfur.

Persistency of volatile sulfur liberation: To determine the tenacity with which the volatile sulfides liberated on heating are retained by the milk, milk was momentarily heated to 90° C. and the volatile sulfides evolved during heating and for each 30 minute period throughout a 4-hour aspiration period were determined. In addition, the sample was checked for intensity of the color produced by the nitroprusside and for cooked flavor at the end of each 30 minutes of aspiration. The average results for two trials are shown in Table 12.

Table 12. Sulfide liberation, nitroprusside test, and cooked flavor of milk momentarily heated at 90° C. and then aspirated continually for four hours*.

Period of aspiration	Volatile sulfur	Nitroprusside	Cooked flavor
Minutes	mg/l	Test	
During heating:	0.333		
30	0.190	4	4
60	0.041	4	4
90	0.036	4	4
120	0.034	4	4
150	0.031	4	4
180	0.025	3	3
210	0.025	3	3
240	0.019	2	2

* Average of two determinations.

The results of this determination show that only a relatively small portion (0.033 mg.) of the total amount of volatile sulfur is evolved during the 15 minutes necessary to bring the sample to 90° C., whereas the major portion is evolved during the first 30 minutes of aspiration. How-

ever, the sample liberated sulfides in decreasing amounts during each subsequent aspiration period throughout the entire four hours, with a small quantity still being liberated at the end of four hours of aspiration. According to these results the method arbitrarily adopted for estimating volatile sulfur actually secured only half of the sulfides made available by the heat treatment. The intensity of the nitroprusside test and cooked flavor were identical after each aspiration period and showed no decrease in intensity until after three hours of aspiration. However, there was a definite nitroprusside test and cooked flavor in milk practically free from volatile sulfides.

The length of time necessary for milk heated to 90° C. to subside in sulfide liberation was also studied. In this experiment, milk was heated to 90° C. within 15 minutes and then immediately cooled. During heating and cooling the sample was well agitated by means of a glass stirring rod. This milk was then stored at 5° C. A two liter sample of this milk was removed, warmed to room temperature, and examined while fresh for sulfide liberation during 30 minutes of aspiration, nitroprusside reaction and cooked flavor. These determinations were also made on the stored milk each day for a period of seven days. These results are shown in Table 13.

These results show that milk stored at 5° C. exhibits a decrease in sulfide liberation. The fresh milk liberated 0.186 mg. of sulfides during 30 minutes of aspiration, and after one day of storage another sample of this same milk liberated 0.117 mg. of volatile sulfur as sulfides. Even after seven days of storage a sample of this milk liberated 0.049 mg. of volatile sulfur.

Table 13. Persistency of sulfide liberation, nitroprusside test, and cooked flavor of stored milk previously heated momentarily at 90° C.*

Age of sample	Volatile sulfur	Nitroprusside	Cooked flavor
	mg/l		
Fresh	0.186	4	4
1 day	0.117	4	4
2 days	0.094	3	3
3 days	0.086	3	3
4 days	0.079	3	2
5 days	0.068	2	2
6 days	0.056	2	2
7 days	0.049	2	1

* Average of two trials.

The intensity of the nitroprusside reaction and cooked flavor was also found to decrease as the storage period progressed. The first change was noted after the second day of storage. The nitroprusside reaction was still definite after seven days of storage, whereas the cooked flavor had practically disappeared.

Influence of Variable Heat Treatments Upon the Labile Sulfur Compounds

Function of time and temperature: Previous investigations have shown that the liberation of sulfides by milk products is a function of time as well as temperature. In these trials the amount of heat labile sulfur, cooked flavor and sulphydryl formation were compared on milk heated to temperatures ranging from 72 to 95° C. for 0 minutes, 30 minutes and 60 minutes. The sulfides were collected during the heating period and for 30 minutes after reaching the desired temperature, the sample having been cooled to room temperature immediately after the desired heat treatment. The trials were conducted and the average results are presented in Table 14.

Table 14. Function of time and temperature upon the heat labile sulfides, cooked flavor and nitroprusside test*.

Temperature	Heating Period								
	Momentary			30 minutes			60 minutes		
	Volatile sulfur	C.F.*	N.P.	Volatile sulfur	C.F.*	N.P.	Volatile sulfur	C.F.*	N.P.
	: mg/l	:	: test	: mg/l	:	: test	: mg/l	:	: test
70	: 0.000	: 0	: 0	: 0.012	: 1	: 1	: 0.019	: 3	: 3
75	: 0.000	: 0	: 0	: 0.032	: 1	: 1	: 0.106	: 4	: 4
80	: 0.031	: 1	: 1	: 0.110	: 3	: 2	: 0.134	: 4	: 4
85	: 0.106	: 2	: 2	: 0.123	: 4	: 4	: 0.158	: 4	: 4
90	: 0.137	: 4	: 4	: 0.167	: 4	: 4	: 0.176	: 4	: 4
95	: 0.157	: 4	: 4	: 0.208	: 4	: 4	: 0.206	: 4	: 4
	:	:	:	:	:	:	:	:	:

* Intensity of cooked flavor (C.F.) or color (N.P. = nitroprusside test); 0 - negative; 1 - slight; 2 - definite; 3 - pronounced; and 4 - strong. These results are on an average of two trials.

These trials demonstrate again that milk does not liberate heat labile sulfides when subjected to momentary heat treatment until a temperature above 75° C. is reached, and that sulphydryl formation and a cooked flavor occur simultaneously with sulfide liberation. However, when milk is heated for a period greater than 30 minutes sulfide liberation, sulphydryl formation and the development of a cooked flavor take place at temperatures as low as 70° C. These results also show that the amount of volatile sulfur liberated, and the intensity of the nitroprusside reaction and cooked flavor development are increased either by a longer period of heat exposure or exposure to a higher temperature. When this milk was momentarily heated to 95° C. 0.157 mg. of sulfur per liter were recovered as heat labile sulfides. However, when this milk was subjected to 95° C. for 30 or 60 minutes approximately identical volatile sulfur values (0.207 mg. per liter), were obtained.

Influence of previous heat treatment: To determine the influence of previous heat treatment upon the formation of heat labile sulfur compounds, milk was subjected to a mild heat treatment previous to determining the volatile sulfides evolved when momentarily heated to 90° C. Samples of milk were subjected to temperatures from 50 to 60° C. for 30 minutes and to 62 to 79.5° C. momentarily and then cooled before determining the volatile sulfides in the usual manner. These samples of milk were also observed for sulfhydryl formation and cooked flavor development. Results are presented in Table 15.

Table 15. Influence of previous heat treatment upon the heat labile sulfides of milk momentarily heated to 90° C*.

Previous heat treatment		After heating to 90° C.**
Temperature	Holding time	
		Volatile sulfur
		mg/l
Control	-----	0.232
50	30 minutes	0.215
55	30 "	0.215
60	30 "	0.225
62.8	Momentary	0.215
68.5	"	0.226
73.6	"	0.218
79.5	"	0.202

* Average of two trials.

** All of these samples had a distinct cooked flavor and gave a strong nitroprusside test.

The heat treatments previous to the volatile sulfide determination ranged from just under the critical temperature to just above the critical temperature. The data in Table 15 indicate that when milk is exposed to temperatures slightly below the critical temperature the subsequent volatile sulfide determination showed only a slight varia-

tion from the normal value. However, when milk is heated to a temperature slightly above the critical temperature the subsequent sulfides evolved show a slight decrease.

Prolonged and high temperature: Prolonged exposure of milk to 95° C. was studied to determine the influence upon the quantity of volatile sulfides liberated during 30 minutes of aspiration after cooling and upon the nitroprusside test, cooked flavor and extent of discoloration. In these trials a 2-liter sample of milk was placed in a 3-liter flask and agitated with a motor-driven glass stirring rod while exposing to 95° C. for the indicated period. Results presented in Table 16 represent the volatile sulfides liberated during 30 minutes of aspiration subsequent to heating and cooling.

Table 16. The influence of heating milk for prolonged periods at 95° C. upon volatile sulfides liberated during a subsequent 30 minutes aspiration period and upon the nitroprusside test, cooked flavor and discoloration.*

Period of exposure to 95° C.	:	Volatile sulfur	:	Nitroprusside	:	Flavor	:	Browning
	:		:	test	:		:	
	:	mg/l	:		:		:	
0 minutes	:	0.158	:	4	:	cooked	:	0
5 "	:	0.165	:	4	:	"	:	0
15 "	:	0.192	:	4	:	"	:	0
30 "	:	0.203	:	4	:	"	:	0
60 "	:	0.208	:	3	:	sl. caramel	:	0
90 "	:	0.091	:	2	:	"	:	1
120 "	:	0.060	:	1	:	"	:	1
150 "	:	0.053	:	1	:	"	:	2
180 "	:	0.012	:	1	:	"	:	3
210 "	:	0.000	:	?	:	"	:	4
	:		:		:		:	

* Average of two trials.

The data in Table 16 demonstrates that an increasing amounts of sulfides are made available by exposing milk to 95° C. for periods of time up to 30 minutes of exposure. However, when the samples were exposed to 95° C. for a period of 90 minutes or longer the amount of sulfide liberated by aspiration are progressively decreased. When milk is held at 95° C. for 210 minutes no volatile sulfides were secured during a subsequent 30 minute aspiration period. In a similar manner, the sulfhydryl groups are shown to decrease with increased period of exposure to 95° C. Milk exposed to 95° C. for periods of from 0 to 30 minutes showed a strong nitroprusside reaction. However, the intensity of the nitroprusside test decreased with increasing periods of exposure. After 210 minutes of exposure the milk itself had become discolored so that it was questionable whether or not the color produced with nitroprusside could be accurately determined.

A strong cooked flavor was observed in the samples held at 95° C. for period ranging from 0 to 30 minutes. After holding the milk at this temperature for 60 minutes or longer a slight caramel flavor similar to that of evaporated milk was observed.

Milk held at 95° C. for 90 minutes or longer took on a brownish discoloration, which increased in intensity with increased periods of exposure. Since the volatile sulfides and, also, the sulfhydryl group were found to decrease simultaneously with the appearance of browning there seems to be a possibility that the occurrence of browning of milk may be associated with the disappearance of the heat labile sulfur compounds.

Additional studies were conducted to determine the influence of prolonged heat treatment of milk placed in varied types of containers. In these trials a portion of the milk was sealed in 18 ounce cans and quart fruit jars and other portions were heated in open 18 ounce cans and in an open flask in which the milk was agitated by a motor driven glass stirring rod. These samples were held at 90° C. for periods from 0 to 240 minutes, then after cooling to room temperature the volatile sulfides were collected during 30 minutes of aspiration. The data are presented in Table 17.

Table 17. Influence of the type of heating vessel upon the sulfides obtained from milk held at 90° C. for different periods of time.*

Period of exposure to 90° C.	Sealed cans	Open cans	Sealed jars**	Agitated in open flask
	mg/l	mg/l	mg/l	mg/l
0 minutes	0.107	0.132	0.125	0.120
30 "	0.088	0.166	0.175	0.170
60 "	0.040	0.178	0.183	0.178
90 "	0.006	0.121	0.129	0.110
120 "	0.000	0.095	0.097	0.093
150 "	0.000	0.064	0.061	0.060
180 "	0.000	0.033	0.031	0.027
210 "	0.000	0.006	0.009	0.006
240 "	0.000	0.000	0.000	0.000

* Average of two trials

**Although these standard fruit jars were sealed in the usual manner, the rubber gasket did not maintain the pressure that developed during heat treatment.

These results show that milk sealed in cans and exposed to a temperature of 90° C. has a relatively small amount of sulfides liberated during a subsequent 30 minute aspiration period in comparison to that heated in other types of containers. The samples heated in sealed cans liberated

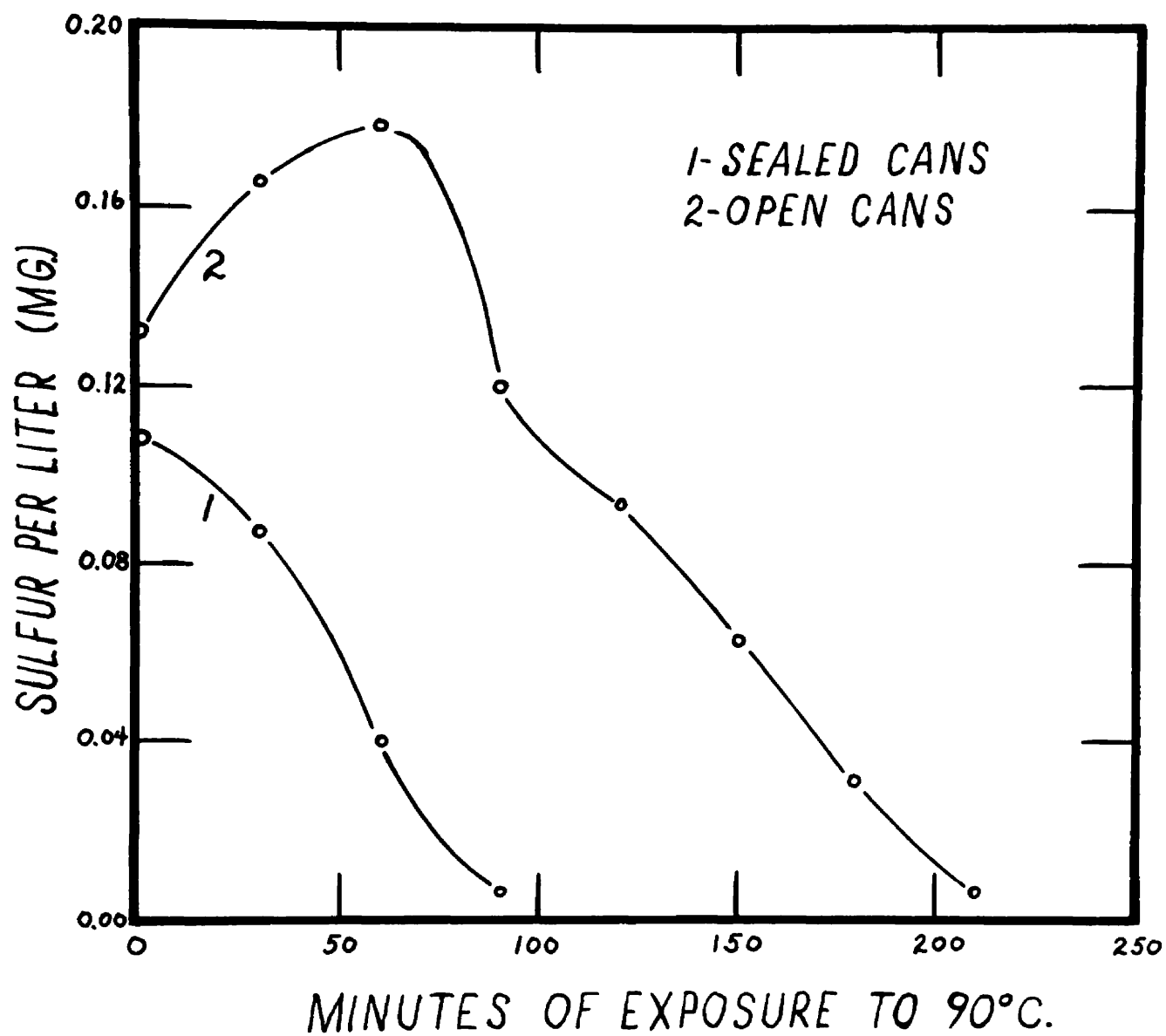


Fig. 5. Results secured by exposing different samples of milk to 90° C. for different periods of time.

only 0.107 mg. of sulfur per liter upon momentary exposure to 90° C., and showed a progressive decrease in amount of sulfides liberated after long periods of exposure. Only a trace of sulfur was obtained in the form of sulfides after 90 minutes of exposure and longer periods of exposure caused a complete disappearance of the volatile sulfides.

Samples heated either in open cans, sealed fruit jars or an open flask with agitation gave practically identical values upon momentary heating, and liberated increasing amounts of volatile sulfur for periods of exposure to 60 minutes. However, longer periods of exposure caused a progressive decrease in sulfides, until no sulfides were liberated after an exposure of 240 minutes. The lack of similarity of values between samples heated in sealed cans and sealed fruit jars indicates that pressure developed in the can during heating plays some significant role in causing the disappearance of the volatile sulfur compounds. These results are graphically portrayed in Fig. 5.

Whey was also found to respond to prolonged heat exposure in a manner similar to milk. Results are presented in Table 18. This indicates that casein is not responsible for the disappearance of the volatile sulfides during prolonged heat treatments.

In other studies, the influence of prolonged heat treatment was ascertained from the standpoint of comparing the amounts of volatile sulfur liberated from milk heated in sealed cans and in sealed fruit jars to temperatures ranging from 90° C. for 0 minutes to 126.6° C. for 30 minutes. In addition these samples were compared for intensity of the nitroprusside test, cooked flavor and discoloration. Results are presented in Table 19.

Table 18. The influence of prolonged heat treatment upon the heat labile sulfur compounds of whey samples held for different periods of time at 95° C.

Period of exposure to 95° C.	Trial 1		Trial 2	
	Sulfur as volatile sulfides*	Nitro-prusside test	Sulfur as volatile sulfides*	Nitro-prusside test
	mg/l		mg/l	
0 minutes	0.125	4	0.122	4
5 minutes	0.135	4	0.132	4
15 "	0.172	4	0.172	4
30 "	0.185	4	0.189	4
60 "	0.186	4	0.180	4
90 "	0.050	2	0.049	2
120 "	0.045	2	0.045	2
150 "	0.037	1	0.036	1
180 "	0.012	?	0.012	?
210 "	0.000	0	0.000	0

* These values were obtained by aspirating the sample for 30 minutes immediately after heating and cooling.

Table 19. Influence of high heat treatment upon the heat labile sulfides of milk.

Heating temperature °C.	Holding period at heating temperature	Observations after heating			
		Volatilesulfur	Nitro-prusside	Cooked flavor	Browning
		mg/l			
Sealed cans					
90	0 minutes	0.110	3	4	0
90	20 "	0.096	3	4	0
108.3	20 "	0.045	2*	4	1
126.6	20 "	0.010	1*	3**	2
126.6	30 "	0.000	1*	3**	3
Glass jars					
90	0 minutes	0.138	4	4	0
90	20 "	0.170	4	4	0
108.3	20 "	0.052	3*	4	?
126.6	20 "	0.012	2*	3**	2
126.6	30 "	0.000	1*	3**	3

* Results not reliable due to off color of milk.

** Not a typical cooked flavor, but a slight caramel flavor.

These results are very similar to those previously reported regarding the influence of prolonged heat treatment upon the volatile sulfides. Milk heated in sealed cans show maximum sulfide liberations when heated momentarily to 90° C., whereas milk heated at 90° C. in sealed glass jars showed maximum sulfide liberation when heated for 20 minutes. However, when milk was heated in either of these containers to temperatures above boiling the amount of sulfides liberated during a subsequent 30 minute aspiration was decreased. Either an increase in temperature or increase in the period of exposure to heat resulted in decreases in sulfide liberation.

The intensity of the nitroprusside test and cooked flavor are shown to decrease slightly with increased browning. The browning prevented accurate observation of the color formation in the nitroprusside test and the flavor developed in the samples heated above boiling was not a typical cooked flavor but more of an "evaporated milk" flavor.

These results demonstrate once more that the volatile sulfides and sulfhydryl groups progressively decrease as the intensity of the discoloration progresses.

Influence of Added Compounds Upon the Heat Labile Sulfur Compounds

Sugars: Quantitative determinations of the volatile sulfides from milk samples containing 5 per cent of added sugar were conducted by momentarily heating the milk to 90° C. Results of three trials are presented in Table 20.

Table 20. Influence of various sugars upon the volatile sulfides liberated from milk momentarily heated to 90° C.

	:	:	:	:				
	:	Control	:	5 % sucrose	:	5 % dextrose	:	5 % lactose
	:	mg/S1	:	mg/S1	:	mg/S1	:	mg/S1
Trial 1	:	0.215	:	0.192	:	0.142	:	0.122
Trial 2	:	0.222	:	0.191	:	0.151	:	0.136
Trial 3	:	0.236	:	0.200	:	0.176	:	0.151
	:		:		:		:	

These results show that 5 per cent sucrose has only a slight retarding influence upon the volatile sulfides evolved from milk momentarily heated to 90° C., but that dextrose and lactose show definite inhibitive properties.

Further studies on the influence of sucrose upon sulfide liberation are presented in Table 21. In these experiments, sucrose was added to milk at the rate of 20 per cent by weight and the volatile sulfides were determined at temperatures ranging from 74 to 90° C.

Table 21. Influence of sucrose upon the sulfide liberation of milk momentarily heated at different temperatures.*

		Sulfur recovered as volatile sulfides	
Temperature		Control	20 per cent sucrose added
		mg/l	mg/l
74		0.000	0.000
76		0.012	0.006
80		0.053	0.019
82		0.082	0.034
90		0.222	0.090

* Average of three trials.

The control sample is shown to evolve 0.222 mg. of sulfur as volatile sulfides when momentarily heated to 90° C., whereas the sample containing 20 per cent of added sucrose evolved only 0.090 mg. of sulfur at this temperature.

These results are portrayed graphically in Fig. 6. Fairly smooth curves are obtained when the amount of sulfides evolved by each series of samples are plotted against the heating temperature. Throughout the temperature range studied, the sucrose is shown to exhibit a definite decreasing action upon the volatile sulfides.

Alcohol: Ethyl alcohol (95 %) was added to milk at the rates of 12.5, 25, and 50 per cent by volume, and the volatile sulfides were determined in the usual manner. The data are presented in Table 22.

Table 22. Influence of alcohol upon the volatile liberation of milk momentarily heated at different temperatures*.

Per cent ethyl alcohol	Sulfur recovered as volatile sulfides	
	82° C.	90° C.
	mg/l	mg/l
0	0.075	0.210
12.5	0.230	0.290
25	0.300	0.310**
50	0.260**	-----

* Average of three trials.

** Considerable alcohol was driven off and some difficulty was encountered in reaching this temperature; therefore, these determinations are not comparable.

These results show alcohol to increase the amount of volatile sulfides liberated. The control samples liberated 0.075 mg. of sulfides at 82° C. and 0.210 mg. at 90° C. Whereas, when 12.5 per cent alcohol was added 0.230 mg. of volatile sulfur was obtained at 82° C. and 0.290 mg. at 90° C.

Some difficulty was encountered in heating samples containing 25 per cent or more of alcohol. In the case of these samples some alcohol was driven over at 82 and 90° C., but was generally withheld from the re-

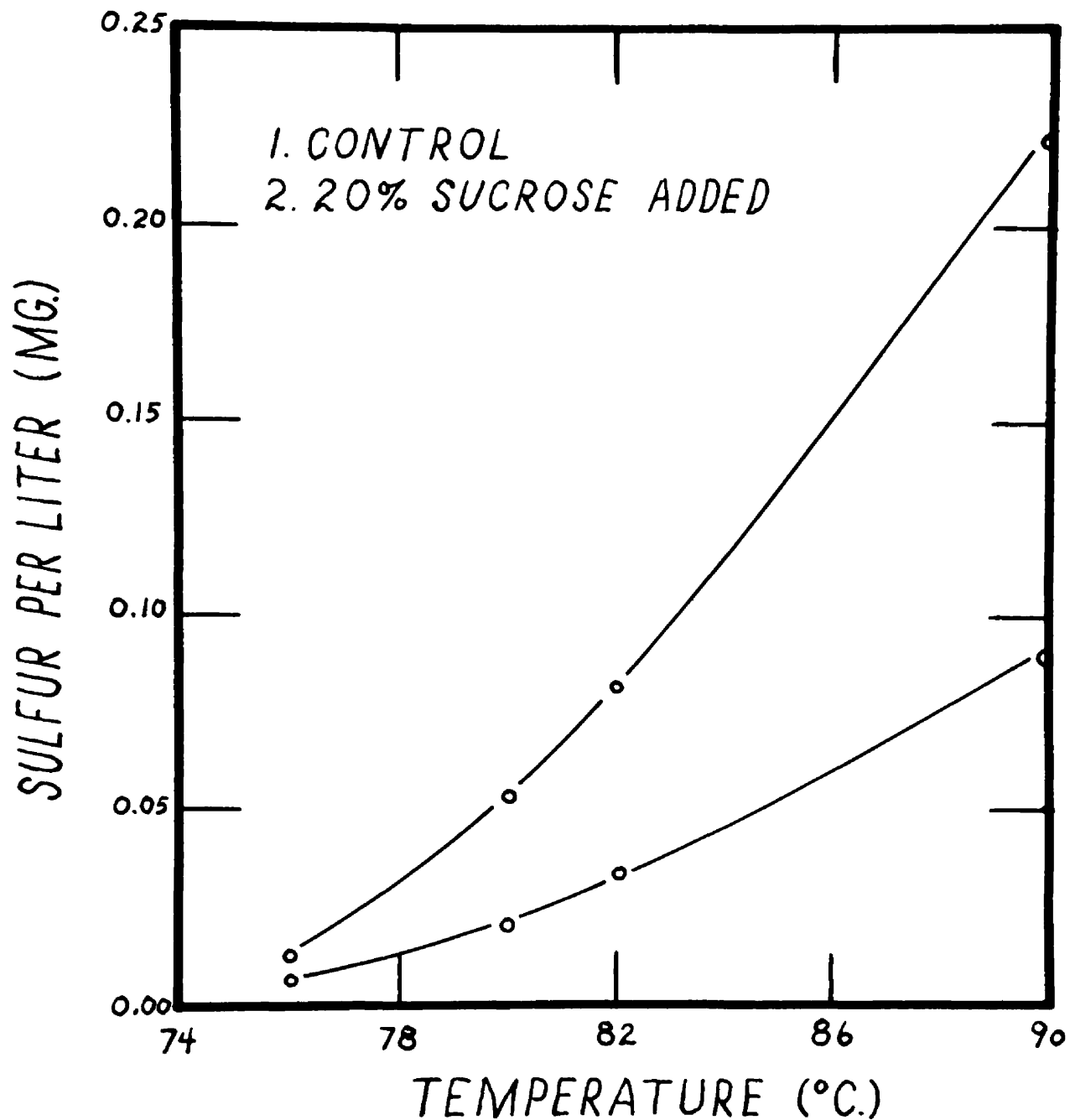


Fig. 6. Influence of 20 per cent sucrose upon sulfide liberation by milk when heated to different temperatures.

ceivers by the foam trap. However, when 50 per cent of alcohol was added to the milk considerable difficulty was encountered in heating the sample to 82° C. within the usual 15 minute heating period and good checks were never obtained due to considerable amounts of the alcohol evaporating over and condensing in the foam trap. For this reason, attempts to conduct the volatile sulfide determination at 90° C. on samples containing 50 per cent of added alcohol were unsuccessful.

The results in Table 22 indicate that even small amounts of alcohol decrease the stability of the heat labile sulfur compounds. In other trials the filtrate obtained by adding 95 per cent alcohol to milk in equal quantities and removing the precipitate, was also found to evolve 0.028 mg. volatile sulfur per liter when momentarily heated to 82° C. This indicates that milk contains heat labile sulfur compounds that are alcohol soluble.

Salt: Sodium chloride was added to milk at rates of 5 to 25 per cent and the sulfides liberated upon momentarily heating the milk to 90° C. were determined. Results are shown in Table 23.

Table 23. Influence of sodium chloride upon the sulfide liberation of milk momentarily heated to 90° C.*

Sodium chloride concentration (per cent)								
0	:	5	:	10	:	15	:	25
:	:	:	:	:	:	:	:	:
Sulfur liberated volatile sulfides								
mg/l	:	mg/l	:	mg/l	:	mg/l	:	mg/l
0.230	:	0.088	:	0.053	:	0.026	:	0.010
:	:	:	:	:	:	:	:	:

* Average of three trials.

These results show that salt exhibits ability to markedly decrease the heat sulfide liberation. In milk heated to 90° C., 5 per cent salt reduced the amount of volatile sulfur to about one-third the normal value. When 25 per cent salt was added only a trace of sulfides were evolved. These results are also shown graphically in Fig. 7.

Cystine and cysteine: Trials were conducted to determine the influence of cystine (Pfanstiehl) and cysteine-hydrochloride upon the volatile sulfides liberated from milk momentarily heated to temperatures ranging from 76 to 90° C. Results are shown in Table 24.

Table 24. Influence of cystine and cysteine upon the liberation of volatile sulfides from milk momentarily heated to different temperatures.

Sulfur liberated as volatile sulfides						
		Cystine per liter			Cysteine-H per liter	
Temperature:	Control:	0.25 gram	0.5 gram		0.25 gram	0.5 gram
	mg/l	mg/l	mg/l		mg/l	mg/l
76	0.012	0.000	0.000		0.020	0.025
80	0.053	0.006	0.006		0.051	0.082
82	0.082	0.009	0.008		0.106	0.158
90	0.222	0.026	0.023		0.232	0.370

These results show that either 0.25 or 0.5 grams of cystine per liter of milk practically but not entirely inhibit sulfide liberation. However, in contrast, the addition of cysteine-hydrochloride to milk definitely increased the amount of sulfides evolved. It may be observed that there is practically no difference in the amount of sulfides secured when either 0.25 or 0.5 grams of cystine per liter were added to milk, but only about 10 per cent of the normal sulfide liberation was secured. However 0.25 grams of cysteine-hydrochloride produced about 35 per cent increase

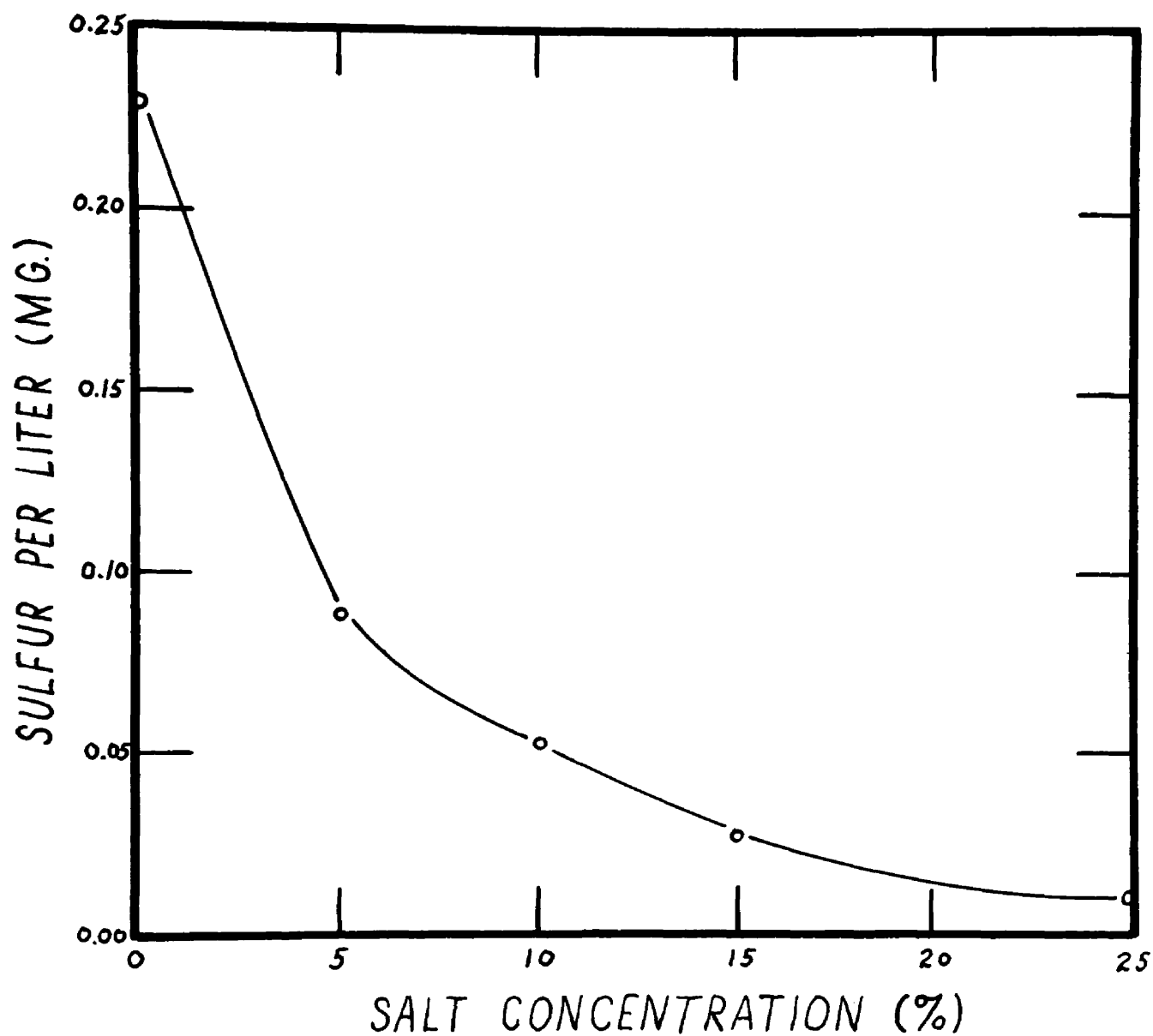


Fig. 7. Influence of salt concentration upon the volatile sulfide liberation of milk momentarily heated to 90° C.

over the normal value for volatile sulfur and 0.5 gram show an increase of approximately 70 per cent in the amount of volatile sulfides evolved.

The results in Table 24 indicating that as the enhancing ability of cysteine on sulfide liberation is due to the action of cysteine on the volatile sulfur compounds in the milk and not to the liberation of sulfides by cysteine itself. This is indicated by the data in Table 25 in which blank determinations of the volatile sulfides liberated by cystine and cysteine in distilled water are presented. When either 0.25 or 0.5 grams of cystine were added per liter of distilled water, no volatile sulfides were evolved even by boiling for 15 minutes. Likewise, when 0.25 gram of cysteine-hydrochloride was used no sulfides were evolved. However, when 0.5 gram of cysteine was added to a liter of distilled water, momentarily heating to 90° C. or boiled for 15 minutes liberated only a trace of sulfides.

Blank trials were also conducted using sodium cyanide and sodium sulrite, and these results are also presented in Table 25. The sodium sulfite solution did not evolve sulfides, but the sodium cyanide solution did produce a slightly positive result indicating that the method used in this study for estimating sulfides is influenced by other reducing compounds.

Reducing substances: Observations presented in the above section indicate that reducing substances enhance sulfide liberation from heat treated milk. Also, Jackson (70) and Gould and Sommer (45) have demonstrated that reducing substances form - SH groups in milk without the application of heat. Therefore, trials were conducted to determine the volatile sulfides of milk when momentarily heated to various temperatures. The results are presented in Table 26.

Table 25. Stability of reagents toward sulfide liberation or formation of reducing compounds when heated in distilled water.

Temperature:	Reducing Compounds as hydrogen sulfide					
	0.5 gm.	1.0 gm.	0.5 gm.	1.0 gm.	0.5 gm.	0.5 gm.
	cystine	cystine	cysteine	cysteine	sodium cyanide	sodium sulfite
° C.	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
80 *	0	0	0	0	0	0
90 *	0	0	0	0.006	0.009	0
100**	0	0	0	0.013	---	---

* Heated momentarily

** Heated for 15 minutes

Table 26. Influence of certain reducing compounds upon the sulfide liberation of milk momentarily heated to 90° C.

Temperature:	Volatile sulfur evolved as sulfides			
	Control	0.5 ppm. of sulfur as sodium sulfide	0.25 gm. sodium cyanide per liter	0.25 gm. sodium sulfite per liter
	mg/l	mg/l	mg/l	mg/l
72	0.000	0.385	----	----
82	0.076	0.555	----	----
90	0.218	-----	0.345	0.395

These results show that either sodium sulfide, sodium cyanide, or sodium sulfite enhance the amount of volatile sulfur compounds evolved from milk. When 0.5 ppm. of sulfur as sodium sulfide was added to milk which was subsequently heated to 72° C., the amount of volatile sulfides evolved were found to be 0.385 mg. per liter; a value less than the amount added to the milk (0.5 ppm. equals 0.5 mg. per liter). However, no volatile sulfides were secured from the control sample heated to 72° C. Also, when the sodium sulfide sample was heated to 82° C., a volatile sulfur value of 0.555 mg. per liter were secured. This value is only slightly less than a

total of the 0.5 ppm. (or 0.5 mg. per liter) added to the milk plus the 0.076 mg. per liter evolved by the control samples at this temperature. Trials involving the use of sodium cyanide and sodium sulfite at the rate of 0.25 gram per liter were found to evolve approximately 70 to 90 per cent more sulfides than the control when heated to 90° C. Data were not secured showing the influence of these compounds when milk was heated at temperatures lower than 90° C.

Hydrogen peroxide: To determine the influence of certain oxidizing agents upon the heat labile sulfur compounds of milk, 30 per cent hydrogen peroxide was added to milk and the volatile sulfides were determined quantitatively in the usual manner. Trials were conducted on samples to which the peroxide was added previous to heating and on samples to which the peroxide was added to the sample immediately upon reaching 90° C. These results are presented in Table 27, and represent the volatile sulfur secured by aspiration during heating and for 30 minutes thereafter, at which time the sample was at room temperature.

Table 27. Influence of adding peroxide before or after heating milk momentarily to 90° C. upon the sulfide liberation*.

Amount of hydrogen: peroxide per liter: of milk	Peroxide added						
	Before heating to 90° C.			After reaching 90° C.			
	Volatile	Nitro-	Cooked	Volatile	Nitro-	Cooked	
	sulfur	prusside	flavor	sulfur	prusside	flavor	
	: test	: test	:	: test	: test	:	
	: mg/l	:	:	: mg/l	:	:	
Control	: 0.236	: 4	: 4	: 0.236	: 4	: 4	
0.25 c.c.	: 0.089	: 3	: 3	: 0.047	: 1	: 3**	
0.5 c.c.	: 0.064	: 1	: 3	: 0.032	: 0	: 3**	
	:	:	:	:	:	:	

* Average of two trials.

** These samples developed a smoky flavor.

The results presented in Table 27 demonstrate that peroxide added either before heating the sample or after the sample reaches 90° C. will reduce the quantity of volatile sulfides evolved and also will decrease the intensity of the color produced with the nitroprusside reagent. When the peroxide was added after the sample reached 90° C. less sulfides were evolved and the nitroprusside test was less pronounced than when the peroxide was added previously to heating. These results show that 0.064 mg. of volatile sulfur per liter was liberated when 0.5 cc. of peroxide per liter was added previous to heating at 90° C., and that only 0.032 mg. were evolved when 0.5 cc. of the peroxide was added after heating the sample to 90° C. The cooked flavor was reduced in intensity only slightly by adding peroxide either before or after heating. When the peroxide was added to the sample after heating the flavor of the milk was not a typical cooked flavor, but can best be described as a smoky flavor.

Further trials were conducted in a qualitative manner in order to determine the amount of peroxide necessary to prevent volatile sulfide liberation and destroy sulfhydryl groups. In this study the peroxide was either added to milk before heating to 90° C. or after heating to 90° C. The volatile sulfides were collected on basic lead acetate paper during a subsequent 30 minute aspiration period. These results are presented in Table 28.

The results of these quantitative trials again demonstrate that peroxide tends to prevent volatile sulfide liberation from milk. More peroxide was required to prevent sulfide liberation if added previous to heating than when added subsequent to heating. For example, the addition

Table 28. Volatile sulfides, nitroprusside test, and cooked flavor of milk treated with different amounts of hydrogen peroxide either before or after heating to 90° C.*

Hydrogen peroxide per liter	Before heating			After heating		
	Volatile sulfides	Nitro- prusside test	Cooked flavor**	Volatile sulfides	Nitro- prusside test	Cooked flavor**
ml						
0.0	4*	4	4	4	4	4
0.25	4	3	4	1	4	4
0.5	3	3	3	0	?	2
1.0	3	2	3	0	?	3
1.5	2	1	3	0	0	3
2.0	2	?	2	-	-	-
2.5	?	?	2	-	-	-
3.0	0		2	-	-	-

* Average of three determinations. All numbers indicate intensity.

**Samples to which hydrogen peroxide was added, either before or after heating, possessed an off flavor best characterized as smoky.

of 2.5 cc. of hydrogen peroxide prior to heating prevented the appearance of sulfides and sulfhydryl groups, whereas, the addition of 0.5 of peroxide per liter of milk previously heated to 90° C. prevented the liberation of volatile sulfides during the subsequent aspiration period. As in previous trials, the addition of peroxide either before or after heating reduced the intensity of the flavor produced by the heat treatment.

pH and Iron Salts: To show the influence of pH upon volatile sulfide liberation, rennet whey was adjusted to various pH values with concentrated ammonium hydroxide or hydrochloric acid. The volatile sulfides liberated by momentarily heating to 90° C. were then determined quantitatively in the usual manner. Also, in this study a series of trials were conducted in which 0.5 ppm. of ferric chloride and 3 ppm. of ferrous chloride were added to whey samples and the influence of pH was studied in the presence of these compounds. These results are presented in Table 29.

Table 29. Influence of pH and ferric or ferrous chloride upon the volatile sulfides liberated by whey momentarily heated to 90° C.

pH	Volatile sulfur recovered as sulfides		
	Control*	0.5 ppm of ferric chloride	3.0 ppm of ferrous chloride
	mg/l	mg/l	mg/l
2	0.026	0.006	-----
3	0.063	0.019	0.005
4	0.097	0.024	0.009
5	0.137	0.125	0.030
6	0.190	0.151	-----
normal	0.202	0.167	0.172
7	0.260	0.230	-----
8	0.330	0.267	0.275
9	0.350	-----	-----
10	0.260	0.208	0.177
10.5	0.010	-----	-----

* Average of three trials.

The data presented in Table 29 show that as the pH of the sample is lowered the quantity of sulfides evolved at 90° C. is decreased. In contrast, an increase in pH above normal to about pH 9 was accompanied by an increase in the quantity of volatile sulfides evolved. However, at pH value above pH 9 (pH 10 and 10.5) a decrease in the amount of sulfides evolved was observed with only a trace of sulfides being evolved at pH 10.5. The results dealing with the influence of pH alone are graphically presented in Fig. 8. Normal whey was found to liberate 0.202 mg. of sulfur as volatile sulfides. At pH 4 this whey liberated only 0.097 mg. per liter and at pH 9 the amount of sulfur liberated as volatile sulfides increased to 0.350 mg. per liter.

The results presented in Table 29 demonstrate that the addition of 0.5 ppm of iron as ferric chloride or 3 ppm of iron as ferrous chloride will reduce the quantity of sulfides liberated by whey; however, the decrease in sulfide liberation produced by these two compounds is only slight.

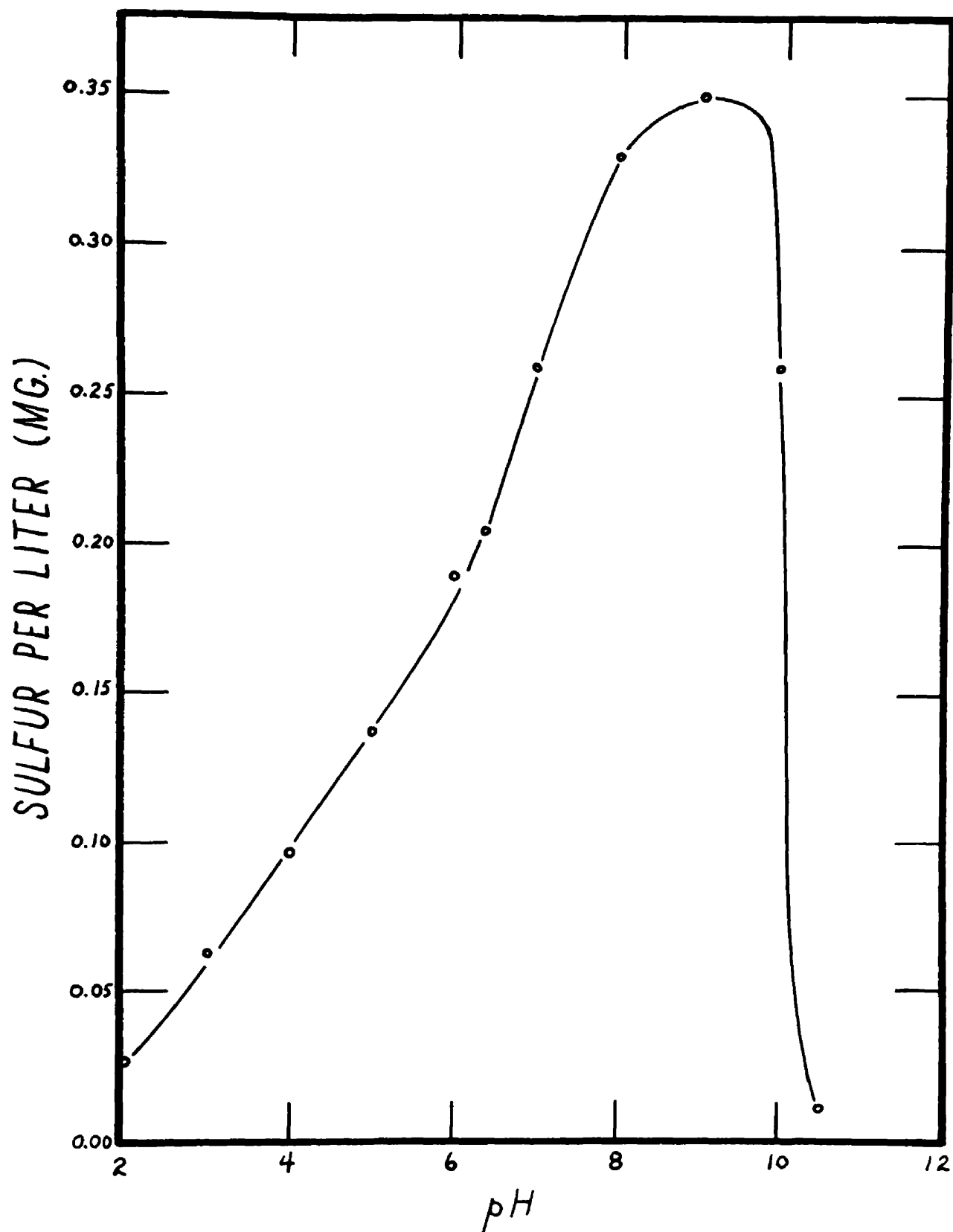


Fig. 8. Influence of pH upon the liberation of volatile sulfides by whey momentarily heated to 90° C.

The form in which the iron is added is apparently of some significance since 0.5 ppm. of iron as ferric chloride was as effective in reducing sulfide liberation from whey heated to 90° C. as 3 ppm. of iron when added as ferrous chloride. The pH of samples is shown to have only a limited influence upon the ability of these compounds to reduce sulfide liberations. At pH values above 8 the ferrous chloride is shown to increase in effectiveness.

Additional studies on the influence of metals: The influence of other metals on sulfide liberation from milk was also studied. The results secured when 0.5 ppm. of copper as copper sulfate is added to milk momentarily heated to temperatures ranging from 74 to 90° C. are presented in Table 30.

Table 30. Influence of added copper upon the volatile sulfides, nitroprusside test, and cooked flavor of milk when heated momentarily at 90° C.*

Temperature	Control		0.5 ppm. of copper added	
	Volatile	Cooked	Volatile	Cooked
	sulfides	flavor	sulfides	flavor
	mg/l		mg/l	
74	0.000	0	0.000	0
76	0.012	?	0.004	0
78	0.016	1	0.006	0
80	0.045	2	0.008	0
82	0.075	2	0.010	?
84	0.095	3	0.012	1
86	0.127	3	0.016	2
88	0.185	4	0.045	3
90	0.235	4	0.075	3

* Average of three determinations.

These results show that 0.5 ppm. of copper practically prevents sulfide liberation from milk until the sample is heated to 86° C. or higher. A cooked flavor was not detected in this milk until heated to a temperature of 84° C., whereas, the control sample developed a cooked flavor when momentarily heated to 76° C. The results presented in Table 30 are also presented graphically in Fig. 9.

Additional trials were conducted to compare quantitatively the influence of various metals upon the sulfide liberation, sulphydryl formation and cooked flavor of milk. These results are presented in Table 31. Copper, mercury and silver are equally effective in retarding sulfide liberation by milk momentarily heated to 90° C. The amount of sulfides liberated by milk is reduced with the addition of increased amounts of these metals. When 1 ppm. was added only a small amount of sulfides were liberated from the milk, but a definite nitroprusside test was apparent and the cooked flavor was pronounced.

Also, in Table 31 additional data are presented showing the influence of ferric and ferrous iron. Although both of these iron compounds decrease sulfide liberation, the ferric iron was more effective. These compounds were found to have only a slight effect upon the nitroprusside test and cooked flavor.

The influence of the negative ion was also studied as a possible factor in the sulfide liberation of milk. Qualitative trials were conducted in which a number of copper salts were added in increasing amounts to milk previous to heating to 90° C. The amount of sulfides liberated were collected on lead acetate paper. The results as shown in Table 32 indicate that the negative ion exhibits no influence upon the amount of

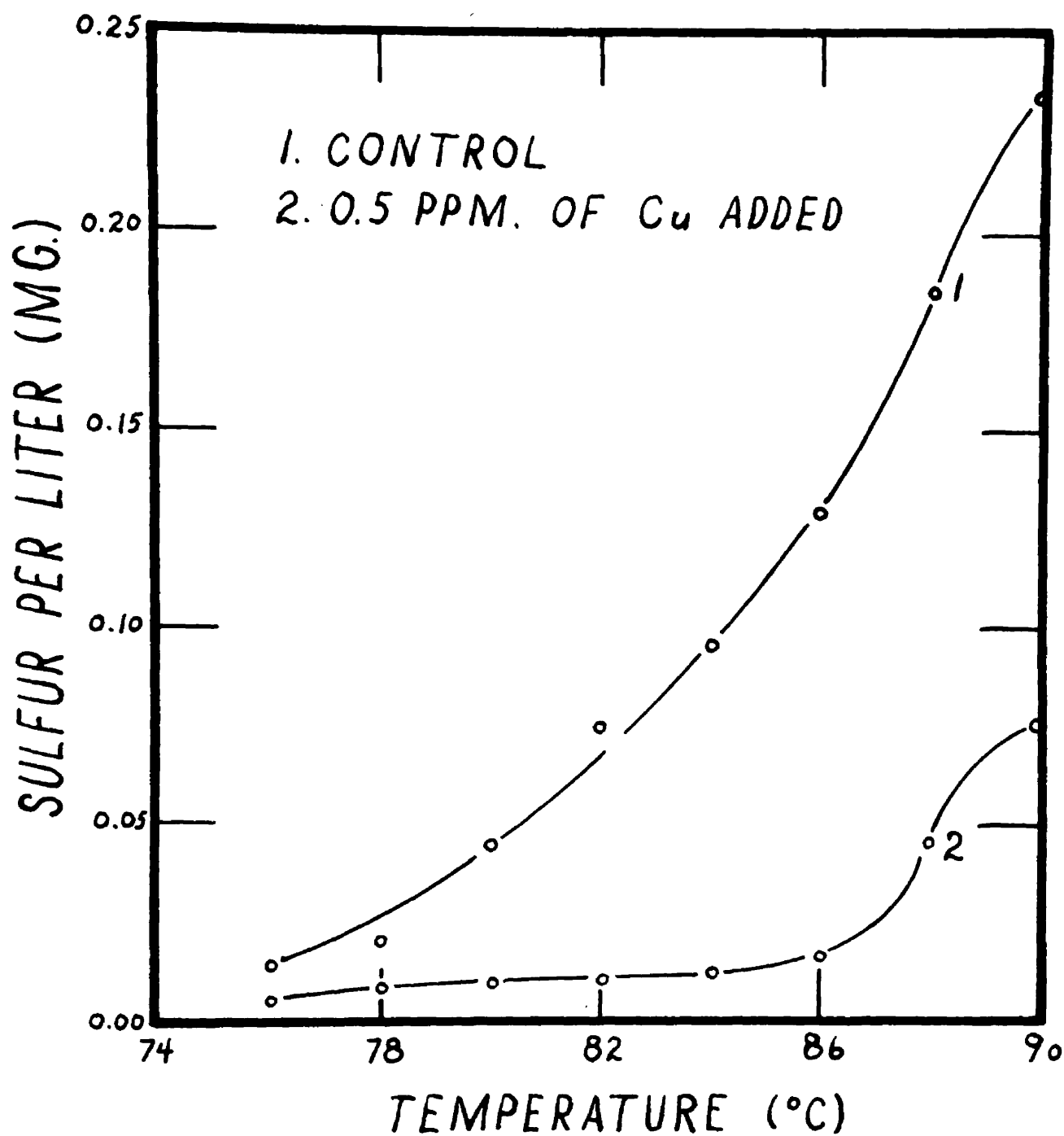


Fig. 9. Influence of 0.5 ppm. of copper upon the quantity of sulfides liberated by milk when momentarily heated to different temperatures.

Table 31. Influence of different metals upon the volatile sulfides, nitroprusside test, and cooked flavor of milk momentarily heated to 90° C.*

Metal salt added before heating	: ppm. of metal	: Sulfur recovered as volatile sulfides	: Nitroprusside test	: Cooked flavor
		mg		
Control	: 0.00	: 0.236	: 4	: 4
Copper sulfate	: 0.50	: 0.032	: 3	: 4
" "	: 0.75	: 0.028	: 2	: 3
" "	: 1.00	: 0.019	: 2	: 3
Silver nitrate	: 0.50	: 0.032	: 3	: 4
" "	: 0.75	: 0.027	: 2	: 3
" "	: 1.00	: 0.019	: 2	: 3
Mercuric acetate	: 0.50	: 0.036	: 3	: 4
" "	: 0.75	: 0.028	: 2	: 3
" "	: 1.00	: 0.019	: 2	: 3
Ferric chloride	: 0.50	: 0.135	: 4	: 4
" "	: 0.75	: 0.090	: 4	: 4
" "	: 1.00	: 0.045	: 3	: 3
Ferrous chloride	: 0.50	: 0.225	: 4	: 4
" "	: 1.00	: 0.174	: 4	: 4
" "	: 3.00	: 0.155	: 4	: 4
" "	: 5.00	: 0.053	: 3	: 3

* Average of two trials.

sulfides liberated. These metals salts were all effective in reducing the intensity of the cooked flavor and of the nitroprusside reaction. When 1.25 ppm. of copper was added to milk previous to heating the intensity of the nitroprusside test and cooked flavor were only slight in the fresh samples and were absent after holding the samples for 48 hours. However, an oxidized flavor developed after 48 hours of storage in the samples containing 1.25 ppm. or more of copper.

In the literature review, certain metals have been reported to react differently when added to the proteins. To ascertain the influence of certain other metals upon the sulfides liberated by heat treated

Table 32. Influence of various copper salts upon the sulfides liberated by milk while fresh and upon the sulfhydryl reaction and cooked flavor of milk after different periods of storage at 5° C.

Compound used	:Amount of :metal used: :(ppm.)	:Sulfides :liberated:	:After storage			
			:Nitroprusside		:Cooked flavor	
			:reaction		:	
			:4 hours	:48 hours	:4 hours	:48 hours
Control	: 0.00	: 4	: 4	: 3	: 4	: 3
Copper nitrate	: 0.25	: 3	: 3	: ?	: 3	: 1
Copper acetate	: 0.75	: 2	: 2	: 0	: 3	: 0
Copper chloride	: 1.25	: ?	: 1	: 0	: 1	: ox**
Copper sulfate	: 2.50	: 0	: 0	: 0	: 0	: ox**

* Average of two trials. The relative intensity is reported by number.

** Indicates oxidized flavor.

milk, trials were conducted in a qualitative manner and are presented in Table 33.

Table 33. Influence of different metals upon the sulfides liberated by milk while fresh and upon the sulfhydryl reaction and cooked flavor of milk after different periods of storage at 5° C.

Compounds used	:Amount of :metal used: :(ppm.)	:Sulfides :liberated:	:After storage			
			:Nitroprusside		:Cooked flavor	
			:reaction		:	
			:4 hours	:48 hours	:4 hours	:48 hours
Control	: 0.00	: 4	: 4	: 3	: 4	: 3
Nickel acetate	: 5.00	: 4	: 4	: 2	: 4	: 2
Stannous chloride	: 5.00	: 4	: 4	: 3	: 4	: 3
Aluminum chloride	: 5.00	: 4	: 4	: -	: 4	: -
Manganese chloride	: 5.00	: 4	: 4	: -	: 4	: -

Table 33 shows that nickel, tin, albumin, and manganese chloride are without material effect upon sulfide liberation, sulfhydryl formation and cooked flavor.

Miscellaneous factors: Since sunlight has been shown to affect the stability of proteins, a study was conducted to determine its influence upon the heat labile sulfides of milk. The milk was exposed to direct sunlight for three hours by placing a 2-liter sample into a 5-liter round bottom flask and agitating with a motor driven glass stirring rod. In addition trials were conducted on milk to determine the influence of formaldehyde, mercurous chloride or iodine upon the volatile sulfides of milk. These results are presented in Table 34.

Table 34. The volatile sulfides liberated by milk exposed to sunlight or treated with formaldehyde, mercurous chloride, or iodine.

Treatment of sample	Sulfur evolved as sulfides at 90° C.		
	Trial 1	Trial 2	Trial 3
	mg/l	mg/l	mg/l
Direct sunlight for 3 hours	0.227	0.220	0.230
Formalin (0.5 cc/l)	0.060	0.055	0.050
Mercurous chloride (0.5 gram/l)	0.012	0.015	0.006
Iodine (0.5 ppm.)	0.082	0.086	-----

These results show that three hours of exposure to direct sunlight does not influence the amount of sulfides liberated by milk momentarily heated to 90° C. However, the addition of small amounts of formalin, mercurous chloride or iodine markedly reduce sulfide liberation.

DISCUSSION

Quantitative studies of the critical temperatures of milk products have pointed toward two general sources of heat labile sulfides. First, the serum proteins are designated as a source of heat labile sulfides by the fact that rennet whey evolves sulfides at the same temperature and in slightly greater quantities than skimmilk. Similarly, buttermilk whey evolves sulfides at a slightly lower temperature and in greater quantities than the buttermilk from which it came. These findings demonstrate fairly conclusively that the serum proteins are a definite source of heat labile sulfides and that casein per se is not a contributor of heat labile sulfide evolved by milk at temperatures ranging from 76 to 90° C.

The fact that cream, buttermilk and buttermilk whey all evolve sulfides at lower temperatures and in greater quantities than either milk, skimmilk or whey demonstrates that the material associated with the butterfat is the second source of heat labile sulfides and the major source of sulfides in products containing rather high concentrations of the material adsorbed on butterfat. In addition, these critical temperature studies have shown that the membrane material is less stable to heat than the other proteins contributing heat labile sulfides.

The lack of heat stability characteristic of the fat membrane is further demonstrated by comparison of the ratio of volatile sulfur to total sulfur and the ratio of total sulfur to total nitrogen of the various milk products. Skimmilk and buttermilk are higher in total sulfur and

nitrogen than milk, whey, cream or buttermilk whey. However, skimmilk liberates fewer volatile sulfides than any of the above mentioned products and buttermilk is excelled in sulfide liberation only by buttermilk whey. This is significant proof that the membrane material is less heat stable than other milk proteins and that casein not only does not contribute sulfides itself, but actually slightly inhibits sulfide liberation. This conclusion is readily apparent when observing that whey and buttermilk whey liberate sulfides in slightly greater quantities than the skimmilk and buttermilk from which they were prepared and that the ratio of volatile sulfur to total sulfur of buttermilk is about twice that of whey.

Washed cream studies indicate that the material adsorbed on butterfat is significantly different in composition to the casein present in milk. The ratio of total sulfur to total nitrogen for washed cream and washed cream buttermilk is about twice that of the cream and buttermilk. However, other data show that the ratio of total sulfur to total nitrogen of whey and buttermilk whey are approximately the same and are about twice that of the skimmilk and buttermilk from which they came. Therefore, it appears that the material associated with the fat phase of dairy products is not especially different in sulfur content from the serum proteins. The trials presented in this paper, however, are not of sufficient number to permit the drawing definite conclusions regarding the composition of the membrane material.

Sharp (137) describes an adhesive material associated with the fat phase of milk as being removed from the fat by high temperature

separation. However, neither three successive washings with distilled water nor high temperature separation produced a significant change in quantity of volatile sulfides secured from cream. This indicates that the precursor of the volatile sulfides is rather firmly attached to the fat globules. Homogenization studies showed the fat membrane and also the other proteins of milk to be unaffected as far as heat labile sulfides were concerned. One might expect a change in critical temperature or quantity of volatile sulfides as Bull (19) has produced denaturation of proteins by shaking. However, Menefee, Overman and Tracy (100) found that homogenization had no significant effect upon the nitrogen distribution of milk.

The fact that milk evolves sulfides after four hours of continued aspiration after heating indicates that volatile sulfides are made available by heat, but that they are not volatilized immediately. This phenomenon is perhaps due to protein rearrangement, as it is unlikely that the volatile sulfides are held in solution during this long period. Also, the fact that aspiration causes a disappearance of volatile sulfides at a much faster rate than the disappearance of sulphydryl groups is a further indication that volatile sulfides are freed by milk proteins for a rather long period after heat exposure. However, storage trials indicate prolonged holding of milk at 5° C. will either allow the sulfides to escape or allow changes to occur in milk whereby the sulfides are recombined with the milk.

The fact that protein rearrangement may occur in milk whereby the volatile sulfides are no longer available is emphasized by studies

showing that the maximum amount of sulfides liberated by heat treatment appear after 30 minutes of exposure to 90° C. After one hour of heating there was no change in the quantity of sulfides available and continued heating was found to cause a gradual disappearance of the volatile sulfides of milk. No sulfides were evolved by milk heated for 3½ hours. This fact can best be explained by suggesting that active groups appear in some milk constituents which combine with the sulfides. The appearance of active groups is indicated by the appearance of a slight discoloration of the milk. Discoloration and the disappearance of volatile sulfides was found to occur in a shorter time when milk was exposed to pressure created by heating in a closed container or by heating in either a closed or open container in an autoclave. In either case it appears that the pressure as well as the temperature evolved played a significant part in the more rapid disappearance of sulfides. Since the volatile sulfides and sulfhydryl compounds were found to disappear from whey in about the same time as from milk when these products were heated for prolonged periods indicate that casein is not necessary for the disappearance or recombination of sulfides.

Studies on the influence of various reagents upon the volatile sulfide liberation by milk demonstrate in a general way that reducing substances enhance sulfide liberation, whereas oxidizing agents prevent sulfide liberation. Undoubtedly this action is due to the greater tendency toward the formation of sulfhydryl groups. Clark (20) was one of the first to demonstrate conclusively that sulfite acted upon cystine to form cysteine.

In general, sugars even in relatively low concentration depress sulfide liberated from heated milk. Sugars having potential aldehyde or ketone groups, such as dextrose and lactose, have a more pronounced depress-

ing action upon sulfide liberation than sucrose. However, 5 or 20 per cent of sucrose did decrease sulfide liberation, but did not influence the critical temperature. The exact mechanism whereby sucrose depressed sulfide liberation is not fully understood. However, sugars are known to exert a definite action toward inhibiting protein coagulation and the decreased coagulation of proteins in the presence of sugar appears to be the explanation for the decreased sulfide liberation.

Sodium chloride and formaldehyde also inhibit sulfide liberation by milk. This, also, may be explained by the depressing action of the compounds upon protein coagulation as has been demonstrated by Klobusitzky (83, 94). In contrast, alcohol is known to enhance heat coagulation and in this study even small concentrations of alcohol enhanced sulfide liberation.

Results of this study indicate that cysteine and certain reducing agents favor sulfide liberation by their action on the milk proteins. Since sodium cyanide and sodium sulfite are known to cause the occurrence of sulfhydryl group in proteins even without the application of heat and, since the compounds are shown to enhance sulfide liberation, it appears that the formation of sulfhydryl groups is the first step in sulfide liberation. Therefore, any method of freeing sulfhydryl groups will enhance sulfide liberation.

Cystine was found to inhibit sulfide liberation. Since Mirsky and Anson (106) have shown that cystine acts as a milk oxidizing agent toward sulfhydryl groups, one may logically conclude that the blocking of free -SH groups will prevent the formation of sulfides. This conclusion would also explain the inhibitive action of hydrogen peroxide upon the

liberation of volatile sulfides. Since peroxide was more effective when added after heating than when added before heating indicates that the peroxide must be available to react with the -SH groups or the sulfides themselves in order to be effective.

Copper, mercury and silver appear to combine with the sulfides liberated by heated milk. These metals retarded sulfide liberation when added at the rate of 0.5 ppm. but did not prevent sulfide liberation until used in concentration of 1.25 ppm. That the metal ion is the active agent is emphasized by the fact that the negative ion was shown to exert no significant influence upon volatile sulfide liberation. Other metals, less active oxidizers than copper, mercury and silver, were shown to be practically without effect upon sulfide liberation.

SUMMARY AND CONCLUSIONS

The heat liberation of volatile sulfides by different milk products was studied quantitatively. When milk products are brought to temperatures of 64 to 80° C. in 15 minutes and then immediately cooled volatile sulfides are liberated abruptly and the amount liberated increases with higher temperatures. By aspirating the sample with nitrogen during heating and for 30 minutes thereafter the amount of sulfide evolved by different milk products was found to vary considerably.

Upon momentary heating volatile sulfides are evolved from milk products as follows:

<u>Product</u>	<u>Critical Temperature</u>	<u>Sulfur evolved as sulfides when heated to 90° C.</u>
Milk	76 to 78° C.	0.240 mg. per liter
Skimmilk	80° C.	0.160 " " "
Whey	80° C.	0.205 " " "
Cream (20 per cent)	70° C.	0.386 " " "
Cream (30 per cent)	68° C.	0.480 " " "
(a) Buttermilk	66° C.	0.512 " " "
(b) Buttermilk whey	64° C.	0.575 " " "

Casein per se plays no significant role in the liberation of volatile sulfides by heated milk. The serum proteins and the material adsorbed on the fat globules are the major sources of heat labile sulfur from milk.

The amount of volatile sulfides liberated by milk when heated to 90° C. shows no relationship to the total sulfur or total nitrogen content of the different milk products.

The material associated with the fat membrane, which is a major source of volatile sulfur, is not removed by washing three successive times with warm, distilled water. This material is not especially high in total sulfur but differs considerably in heat stability from the other constituents contributing volatile sulfides.

The amount of sulfides evolved by milk are decreased by continued aspiration or during storage at 5° C.; however, a small amount of sulfides are evolved by milk after 4 hours of continued aspiration or after 8 days of storage at 5° C.

The temperature of separation, homogenization at 2,500 pounds pressure, previous heating to temperatures below the critical temperature, or exposure to direct sunlight have no influence upon the temperature at which sulfides are liberated by milk products.

The milk from Guernsey cows is slightly higher in total and volatile sulfur than the milk from Holstein cows. The amount of sulfides evolved by milk and the total sulfur content was found to increase as the lactation period progressed.

When milk is heated at 90° C. the amount of sulfides evolved increases with an increase in the time of exposure up to 30 minutes. However, after 1 hour of exposure the amount of volatile sulfides decreases until no sulfides are evolved by milk held $3\frac{1}{2}$ hours, at which time the milk was also negative to the nitroprusside test, had a caramel flavor and a brownish color. Milk held in sealed cans at 90° C. for $1\frac{1}{2}$ hours did not evolve sulfides.

Milk autoclaved in sealed cans or glass jars for 30 minutes at 126.6° C. did not evolve sulfides or contain sulfhydryl groups.

Five per cent of sucrose, dextrose or lactose added to milk reduced the quantity of sulfides liberated. The addition of 20 per cent sucrose had no influence upon the temperature at which sulfides are liberated, but decreased the quantity by 50 per cent.

Increased concentrations of sodium chloride progressively decreases the quantity of heat labile sulfides liberated by milk. When a concentration of 25 per cent was used practically no sulfides were evolved.

The addition of 10 to 20 per cent of alcohol increases volatile sulfide liberation.

The addition of a small amount of cystine to milk inhibits sulfide liberation, whereas, the addition of a small amount of cysteine enhances sulfide liberation.

Sulfide liberation is increased by adding small amounts of sodium sulfide, sodium sulfite, or sodium cyanide.

Sulfide liberation is increased by raising the pH above 6.5 and decreased by lowering the pH below 6.5.

Sulfide liberation by heated milk may be prevented by adding a small amount of hydrogen peroxide, formaldehyde, mercurous chloride or as much as 1.25 ppm. of either copper, mercury, or silver as a salt. These compounds are more effective when added after heating. Iron has only a limited ability to decrease sulfide liberation and tin, aluminum, maganese, and nickel are without effect.

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