THE EFFECT OF HEAT TREATMENT ON THE DISTRIBUTION OF RESIDUAL AND ADDED COPPER IN FLUID MILK SYSTEMS

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John Samuel Sargent

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Major professor

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THE EFFECT OF HEAT TREATMENT ON THE DISTRIBUTION OF RESIDUAL AND ADDED COPPER IN FLUID MILK SYSTEMS

By

John Samuel Sargent

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ABSTRACT

Whole fluid milk and model milk systems containing from 100-500µg added Cu/l were subjected to various heat treatments in an attempt to investigate the mechanism by which heat imparts resistance to copper-catalyzed lipid autoxidation in whole fluid milk. The total copper content of centrifugally separated cream and skimmilk fractions from 100 ml samples of milk was determined by a carbamate method utilizing freeze-drying as a step in the analytical procedure. This innovation permitted multiple experimentation on a common sample of milk.

Data from experiments with whole milk and model milk systems indicated that added copper is preferentially bound to skimmilk proteins. Storing raw whole milk treated with 100-500µg Cu/l at 34 F for 18 hr resulted in migration of 2-4% of the added copper to the cream phase. Heating this milk to temperatures greater than 140 F increased the copper content of the cream phase by as much as 600%. The greatest change in extent of heat-induced copper migration to the cream occurred during heat treatments from 165-175 F. Milk having a total copper content of 40-240µg Cu/l exhibited maximum and constant adsorption of copper by the fat fraction following both flash and 10 minute holding at 185-200 F. When the total copper content of the milk was increased to 500-700µg Cu/l, maximum copper adsorption occurred in the cream following both flash heating at approximately 180 F

and 10 minute holding at 170 F; heating the milk to higher temperatures resulted in desorption of copper from the cream phase. Supplemental data from washed cream experiments indicated that heat-induced adsorbed copper was most tenaciously bound to the cream phase in whole milk subjected to momentary heating at 180 F.

A series of experiments was performed on model milk systems comprised of washed raw cream and a milk dialyzate dispersion of centrifugally separated micellar casein, the whey protein-bearing supernatant from micellar casein, or sodium caseinate. The different heat-induced copper migration patterns obtained from these milk systems suggests that their individual copper-protein complexes have different reactivities and stability and the overall pattern exhibited in a given milk system is a function of temperature, time of heating, fat globule interfacial area, protein composition and quantity. The observation that the heat-induced copper adsorption-desorption phenomenon occurs in the cream phase of a milk system devoid of skimmilk proteins suggests that this pattern is solely characteristic of the cream fraction.

The addition of the sulfhydryl group blocking reagent, N-ethylmaleimide prior to heating of whole milk or model systems resulted in partial inhibition of copper adsorption by the cream phase at all temperatures of heating. Similar results were obtained when Iodoacetamide was added prior to

heating of a model system containing sodium caseinate. The fact that the presence of N-ethylmaleimide partially inhibits copper migration to the cream in a milk system with a protein-free aqueous phase, suggests that copper is complexed in some fashion by heat activated sulfhydryl groups in the fat globule membrane as a result of heat treatment. Indirect evidence suggests that possibly the copper-protein complex(es) adsorbs at the fat globule interface in a manner that inhibits the catalytic ability of copper to breakdown hydroperoxides to free radicals.

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CONTENTS

FIGURES

Figure Page

INTRODUCTION

Milk lipids are recognized as a major contributor in determining consumer acceptability of most dairy products. However, the lipid material in dairy products, like most food fats of vegetable and animal origin, is rather sensitive to deterioration by oxidative processes. Much data have been gathered on the conditions which initiate and accelerate these reactions. The role of metals such as copper and iron in accelerating lipid autoxidation by catalyzing the breakdown of hydroperoxides to free radicals is well known. Likewise extensive research has demonstrated that heat treatment of milk to temperatures greater than 165 F causes the liberation or "activation" of sulfhydryl groups in the non-casein protein fraction. The appearance of such available sulfhydryl groups coincides with increased antioxigenic properties of milk. Exactly why heat treatment increases the resistance of milk lipids to oxidation is not known.

This investigation of the effects of heat treatment on the distribution of residual and added copper in fluid and model milk systems was performed in an attempt to elucidate the mechanism of heat-induced resistance to metal catalyzed autoxidation. In addition, sulfhydryl group blocking agents were employed in milk systems for the purpose of characterizing a possible interrelationship between heat activated sulfhydryl groups and copper catalyzed autoxidation of milk lipids.

REVIEW OF LITERATURE

The impairment of flavor quality as a result of autoxidation of milk lipids in dairy products is widespread and manifests itself in many ways. No other chemical deterioration has been studied so extensively as autoxidation, nor has any other problem probably been investigated from so many different aspects. No attempt has been made in this thesis to exhaustively review the literature unless it pertained to the distribution of natural and added copper in whole fluid milk or to the role of copper in the catalysis of development of oxidized flavor in fluid milk. More thorough treatment on the autoxidation of dairy products is included in reviews by Brown and Thurston (7), Day (10), Greenbank (27), Kruisheer (45), Pont (64), Riel and Sommer (66), Stull (72), and on lipids in general by Holman (34), Morris (57) and Lea (52).

A. Copper in Whole Fluid Milk.

1. Level of Natural Copper.

Milk taken directly from the cow contains a low but variable concentration of natural copper. Earlier works (9, 13) reported values ranging from 200-800µg Cu/l of uncontaminated raw milk, while more recent research (16, 36, 45) indicated a copper content of 30-200µg Cu/l of fresh raw milk. The difference is attributed to gradual improvements in technique of analysis and sampling (9).

Koppejan and Mulder (43) and Menger and Mulder (54) showed clearly the variations in copper content that may be expected in milk samples taken from individual cows at different stages of lactation. Natural copper is present in high concentrations (up to 200µg Cu/l) during the first days of a lactation period but gradually declines to normal levels (20-40µg Cu/l) during the first two months of lactation.

The natural copper content of milk generally is not considered to be influenced by dietary copper (18, 54). However, Davis (12) stated that the copper content of milk can be almost doubled by a large increase in the dietary intake by the cow. King and Dunkley (45) increased the natural copper content of cow's milk by drenching the cow with 10 g doses of copper sulfate.

2. Physical State of Copper in Milk.

In an early study, Rice and Miscall (65) concluded that copper in milk was in the ionic state. Results of later investigations (1, 40) indicated that copper does not exist as such, but is complexed with the lipo-protein layer surrounding the fat globules. Davies (13) presented data from diffusion experiments which demonstrated that the amount of ionic copper in milk was negligible at the normal pH of milk; however, the concentration of ionic copper increased with a decrease in pH.

King et al. (40) found natural and added copper associated with the fat globule membrane in a non-dialyzable state. Added copper in the skimmilk was slightly dialyzable; the amount of unbound metal increased as the pH was lowered to 3.0.

Diehl (14) postulated that copper forms a complex with amino and hydroxyacids through coordinating groups so as to form chelate rings. This type of complex would inhibit the ionization of copper in fluid milk.

3. The Distribution of Copper in Milk.

Many investigators have studied the partition of copper between skimmilk and cream; however, the data are both incomplete and controversial. Some workers have reported that added copper is preferentially adsorbed by the fat phase of the milk while others believe copper is primarily associated with the skimmilk.

Rice and Miscall (65) found that copper was uniformly distributed in milk in accordance with total nitrogen or water content. Davies (13) reported added copper was uniformly distributed in gravity-separated milk, but was concentrated in the cream of milk separated by centrifugation. He concluded that added copper was distributed in proportion to the surface area of the fat globules.

Willard and Gilbert (81) reported that copper became

concentrated in the cream of milk subjected to contamination from copper-alloy equipment. Miller and Tracy (55) recovered most of the copper from the skimmilk when cream used in a continuous butter-making operation was exposed to heavy copper contamination. According to data of Hartman (30), added copper was preferentially adsorbed by the whey proteins.

By fractional precipitation with ammonium sulfate, Dills and Nelson (16) isolated a copper containing protein (0.19% Cu, 15% N) from skimmilk. This fraction had no ascorbic acid oxidase activity and the copper was non-dialyzable at pH 6.5. No attempt was made to relate this copper-proteinate to haemocuprein (0.34% Cu) in blood.

Allan (1) studied the kinetics of ascorbic acid oxidation in milk and butter serum. He concluded that both added and natural copper were nonionic and were associated with the fat globule membrane.

Koppejan and Mulder (43) investigated the variation and distribution of natural copper in individual milks. They found that 4-43% of the total copper was associated with the fat globules. Their limited data indicated that added copper does not accumulate at the fat globule surface. Menger and Mulder (54) likewise reported that added copper became primarily associated with the skimmilk proteins, and that natural copper was primarily associated with the fat globule membrane.

In a more recent investigation, King et al. (40) employed a radioactive tracer to determine the distribution of copper in milk. Naturally occurring copper was concentrated (ca. 10-35% of the copper) at the surface of the fat globules; whereas, most of the added copper was uniformly associated with the skimmilk proteins. Only 2-3% of the total added copper was associated with fat globules. Natural and added copper in association with the fat globules were recovered with the fat globule membrane proteins.

King and Williams (41) employed Cu^{64} to determine the distribution of natural copper in milk at intervals during early lactation. The amount of copper associated with fat globules was about 15% of the total copper 2-4 weeks after parturition and 35% after 10 weeks. A combination of cream washing and chelation by ethylenediamine tetraacetic acid (EDTA) removed up to 90% of the lipid-bound copper. However, following mixing of this washed cream with the original skimmilk and reseparation, the copper content of the cream increased. Administration of the isotope by infusion or by drench yielded similar result with respect to distribution, but the rate of appearance in milk was slower by drenching.

B. Copper Analysis of Milk and Milk Products.

Many modifications of the carbamate procedure (17, 31, 32, 44, 53, 55, 61, 74, 83) and dithizone procedure (35, 58) have been applied to the analysis of copper in dairy products.

Gehrke (22) developed a spectrographic method for determining the copper content of milk and dairy products. Procedures for the simultaneous microdetermination of copper and iron in biological systems are also available (50, 84, 85, 86).

Mulder and Koppejan (43) proposed a mathematical calculation of the quantity of copper present in milk plasma and the quantity of copper retained by the butterfat globules. They based their calculations on the assumptions "that copper is equally distributed over all globules and that the difference in size between the fat globules may be neglected. The calculation was carried out as follows:

Suppose that 1 g of plasma is found to have 'a' µg of copper, and the 1 g of butterfat globules retains 'b' µg of copper. When the fat content of the initial milk is 4%, and that of the cream is 20%, and of its skimmilk 1%, the following equation will result:

Copper in 1 kg of milk = 40b + 960a
Copper in 1 kg of cream = 200b + 800a
Copper in 1 kg of skimmilk = 10b + 990a.

The values of 'a and 'b' can be calculated from these equations."

These investigators disregarded the immense surface area presented by the small fat globules present in the skimmilk fraction.

C. Copper Catalysis of Lipid Autoxidation in Fluid Milk.

1. Solubility of Copper in Milk.

Miscall et al. (56) and Gebhardt and Sommer (21) investigated the factors affecting the solubility of copper in fluid milk. An increase in titratable acidity of milk decreased the solubility of metallic copper. Removal of milk gases or addition of carbon dioxide decrease the ability of milk to dissolve copper, whereas, the presence of oxygen in milk increased the solubility of copper (21, 56). Maximum solubility of copper in milk occurred following a 30-120 minute exposure to copper at 158 F (21) or flash heating to 140 F (56). The solubility of copper at the boiling temperature of milk was of the same magnitude as that observed at room temperature. Preheating of milk to various temperatures above 158 F decreased the solubility of copper in milk. This effect of preheating was greater as the temperature and time of preheating was increased (21). Pasteurized milk was shown to dissolve more metallic copper than raw milk at the same temperature (56).

2. Source and Degree of Copper Contamination.

The first milk which passes through dairy plant processing equipment after cleaning and sanitizing treatments is most heavily contaminated with metals. Explanations proposed for this greater initial contamination are based on the formation of a protective coating during product flow

that retards solution of the metal (11, 15) and on the formation between processing runs of a film of soluble metal oxides this is more easily removed by the first milk (79).

Krukovsky and Guthrie (46) found that 0.1 ppm of added copper caused tallowy flavors in milk not depleted of its ascorbic acid when held for 24 hours at 0-5 C. Williams and Burgwald (82) induced the typically oxidized flavor in fluid milk with the addition of 2 ppm of copper after pasteurization.

Pont (63) reported that the addition of 1.0 ppm of copper produced a well-defined oxidized flavor in pasteurized whole and skimmilk following storage at 3 C for 2-3 days. Under identical conditions, 0.1 ppm of copper produced a slight off-flavor described as "cardboard" in whole milk. Hollander and Tracy (33) produced an oxidized flavor in dried whole milk when as little as 0.5 ppm of copper was added during the preheat treatment of the milk.

King and Dunkley (41)(45) observed a highly significant correlation between the concentration of natural copper in milk and the incidence and intensity of spontaneous oxidized flavor. They concluded that copper present in milk as it comes from the cow is an important catalyst of oxidized flavor.

3. Ascorbic Acid.

This compound plays an ill-defined role in the

development of copper-catalyzed oxidized flavor in fluid milk. Considerable experimentation has been performed in an attempt to elucidate its contribution to the off-flavor in whole milk (2, 5, 19, 20, 29, 46, 47, 48, 50, 62, 63, 69, 71, 72, 80, 82).

Guthrie and Krukovsky (28, 46) made the interesting observation that milk was protected against oxidized flavor when all of the reduced ascorbic acid was destroyed. These findings were confirmed by Tobias and Herreid (78); in addition, they noted that a degradation product of ascorbic acid, gulonic acid, was also destroyed before inhibition of oxidized flavor was obtained. With the destruction of this degradation product, oxidized flavor did not develop for five to seven days in milk samples treated with 1 ppm copper.

In a later publication, Krukovsky and Guthrie (47) presented data indicating that the oxidation of the lipid fraction of fluid milk is coupled to that of ascorbic acid when a certain equilibrium between the reduced and oxidized forms has been established. They attributed the protective influence of large amounts of ascorbic acid added to milk to the exhaustion of dissolved oxygen prior to the establishment of the favorable equilibrium between the oxidized and reduced forms of Vitamin C.

Smith and Dunkley (69) concluded that at the copper

concentrations normally present in milk, ascorbic acid is an essential reactant in spontaneous, as in copper-induced, oxidized flavor. At copper levels greater than 1.0 ppm, ascorbic acid was not necessary for lipid peroxidation, presumably because at higher copper/protein ratios, the protein rather than ascorbic acid reduces copper to the cuprous state (69). The pro-oxidative activity of ascorbic acid may depend upon its ability to reduce copper to its lower valence and to form a specific association with copper which in some unexplained manner increases the pro-oxidative properties of the milk (42).

4. Processing.

a. Separation.

Roland and Trebler (67, 68) reported that the sensitivity of standardized milk and cream to copper-induced oxidized flavor appears to be related to fat content. Skim-milk exposed to metallic copper failed to produce the typical oxidized flavor. Mechanical separation of milk produced a marked decrease in sensitivity to copper-induced oxidized flavor.

b. Homogenization.

Homogenization retards the development of copper-induced oxidized flavor (51, 76, 77).

Thurston (77) postulated the resistance of homogenized

milk to develop oxidized flavor is due to increased adsorption of protective protein on the surface of the fat globule. Larsen et al. (51) reported the mechanism by which homogenization prevents or retards development of oxidized flavor does not appear to be associated with the redox potential of the milk.

The migration of unstable lipid components, presumably phospholipids, from the fat globules into the serum phase as a result of homogenization has been suggested as a possible explanation for the retardation of oxidized flavor in homogenized milk (49, 76). King (38) recently proposed that homogenization induces an irreversible change in the structural configuration of the copper-protein-lipid complex in such a way that ascorbic acid is no longer able to initiate the formation of lipid free radicals.

An investigation by Tarrassuk and Koops (75) has shown the concentration of the phospholipids and the copper-protein complex per unit of surface area to be decreased proportionately to the homogenization pressure. They concluded this decrease in concentration per unit of newly formed fat globule surface appears to be the most important factor that retards the development of oxidized flavor in homogenized milk.

c. Heat Treatment.

The addition of 2.5 ppm of copper to milk follow-

ing pasteurization tends to cause a more frequent and more intense development of oxidized flavor than does contamination with identical amounts of copper prior to pasteurization (3, 4, 23). Similar results were obtained when copper was added to whole milk prior to spray drying (70).

Gould and Sommer (24) and Josephson and Doan (37) demonstrated that heat treatment of milk at temperatures high enough to produce cooked flavor resulted in milk more resistant to oxidation. This was attributed to production of volatile sulfides or sulfhydryl compounds which could serve as antioxidants.

Gould and Sommer (24, 25) reported a correlation between the development of cooked flavor and oxidized flavor in whole milk. The temperature range at which cooked flavor appeared was 167-172 F for momentary heating and 158-162 F for a 30 min. holding time. The momentary heating required to produce the cooked flavor was raised to 183-187 F when 1 ppm copper was added after heating. When 1 ppm of copper was added to the milk prior to momentary heating to 183-187 F no oxidized flavor developed in the milk; heating below these temperatures did not prevent the development of oxidized flavor. Copper added to milk following heating accelerated the development of oxidized flavor in all instances, even when the milk was heated to 194 F.

Brown and Olsen (8) performed experiments with washed cream and found that heating of the milk at 180 F for 5 min. prior to washing of the cream did not affect the susceptibility of the cream to oxidized flavor when contaminated with copper.

Forrester and Sommer (19) attempted to relate the oxidation of milk protein and susceptibility of milk to oxidized flavor development. They postulated that ascorbic acid may serve as a hydrogen carrier in the oxidation of lipids and proteins, and that reactive sulfhydryl groups must be present to initiate protein oxidation. A heat treatment of 170-180 F for 5-10 min. would initiate more active sulfhydryl groups for the oxidation of milk proteins. The available sulfhydryl groups would exhaust the oxygen present in the milk, thereby preventing lipid oxidation with its accompanying flavor development. Copper in the cuprous state promotes oxidized flavor by blocking the reactive sulfhydryl groups so that protein oxidation cannot proceed (19, 26). The amount of copper required to initiate development of the flavor would be related to the number of free sulfhydryl groups in the milk protein. Such a postulation is one attempt to explain why the concentration of copper which must be added to produce an oxidized flavor varies so greatly from one milk to another (19).

In a study of the distribution of added copper and iron

in fluid milk systems, Stine (70) demonstrated that heat treatment of milk had a pronounced effect on the displacement of iron from the fat globule interface. Data obtained from experiments wherein copper was added to milk prior to heat treatment were more inconclusive, ranging from positive to negative displacement of copper from the interface. These data were calculated using the simultaneous equations of Koppejan and Mulder (43) and possibly, the inconsistencies noted may be due in part to the assumptions and limitations of these equations.

A thorough search of the literature available failed to disclose other possible effects of heat on the distribution of natural and added copper in milk.

EXPERIMENTAL METHODS AND MATERIALS

A. Whole Milk Systems.

1. Sampling and Preparation of Milk for Heat Treatment.

Individual raw milk samples were obtained following the milking of cows picked at random from the University Holstein, Jersey and Brown Swiss herds. In order to avoid copper contamination, the milk was obtained directly from milking parlor glass "weigh jars" and delivered to four liter erlenmeyer flasks that had been scrupulously cleaned with concentrated nitric acid. (See Appendix). The milk was tempered to 80 F and treated with standard copper solution (Appendix) in amounts ranging from 100 to 500 µg Cu/l of milk.

2. Heat Treatment.

Representative control and copper treated milk samples in volumes slightly in excess of 100 ml were delivered to a two-neck 300 ml round bottom flask fitted with standard taper adapters and an immersion thermometer. The milk was then flash heated under constant agitation in a boiling water bath to temperatures of 140 to 200 F in 5 and 10 degree increments. The heated milk was either cooled immediately or maintained at a controlled temperature for 10 minutes before cooling to 80 F in an ice bath.

3. Separation.

A 100 ml aliquot of the cooled milk was accurately

pipeted into 125 ml International Centrifuge separatory funnels and either separated immediately at 270 rcf for 1 hour at 34 F, or stored 18 hours at this temperature prior to separation. The skimmilk fractions were delivered to 250 ml beakers and the cream fractions were quantitatively rinsed from the separatory funnels into 150 ml beakers with three 30 ml aliquots of redistilled water (conductively less than 0.9 μ mho) tempered to 120 F. Representative samples of the copper treated whole raw milk and skimmilk fractions were analyzed for fat content by the Roese-Gottlieb method (Mojonnier modification).

4. Copper Determination.

The skimmilk and cream fractions were frozen to -30 F for 12 hours and subsequently freeze-dried for 48 hours in a Stokes Freeze Dryer at a pressure of 80-100 microns of mercury with water at 110 F circulating in the plates. The freeze-dried material was removed quantitatively from the beakers with the aid of a polypropylene spatula and delivered to 20 ml platinum crucibles. The material was ashed in a muffle furnace at 550 C for 10 hours and the copper content of the resulting ash determined colorimetrically by a carbamate method (39) (See Appendix) employing carbon tetrachloride as the extracting solvent for the copper-carbamate complex.

B. Model Systems.

1. Milk Dialyzate.

Strips of "Visking" seamless cellulose dialysis tubing (1 7/8" diameter) were filled with 1500 ml volumes of redistilled water. The sealed dialysis membranes were then supported in stainless steel perforated cheese strainers and completely immersed in milk (33-35 F) stored in a farm bulk-milk tank. The water was allowed to equilibrate with the bulk milk supply under intermittent agitation for a period of 36 hours.

2. Washed Cream.

Five gallons of Brown Swiss milk were centrifugally separated in a laboratory model De Laval cream separator. The cream was washed with equal volumes of distilled water at 120 F and reseparated a total of five times.

3. Micellar Casein.

Fresh whole milk was obtained from individual Brown Swiss cows by the sampling method previously described. One liter volumes of the milk were centrifuged at maximum speed in an International Centrifuge at 34 F for 1 hour. The skimmilk fraction was siphoned into polypropylene centrifuge vessels and spun in a Beckman Model L preparative centrifuge at 41,000 rcf for 6 hours at a temperature of 32-40 F. The supernatant liquid containing the whey proteins was decanted and stored at 34 F for use in a model milk system. The remaining micellar casein pellets were dispersed in

fresh milk dialyzate (32-40 F) by high speed mechanical agitation for 20 seconds. The redispersed micellar casein was made up to a volume of 1 l. with fresh milk dialyzate. This dispersion was then centrifuged in the preparative centrifuge as previously described for the original skimmilk. The casein pellets were once again dispersed in fresh milk dialyzate and reprecipitated at 41,000 rcf. Following the second washing, the micellar casein pellets were dispersed by mechanical agitation in 900 ml of fresh milk dialyzate in a cold room at 34 F for 24 hours. This final dispersion was filtered prior to incorporation in a model milk system.

4. Purified Casein.

Sodium caseinate was prepared from isoelectric casein obtained by a method described in Biochemical Preparations (8a) (See Appendix).

5. Beta-Lactoglobulin (3X) was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

6. Treatment of Model Milk Systems.

The prepared model systems were flash heated, cooled, separated, and the copper content of the subsequently freeze-dried fractions determined in the manner described for whole milk systems. The reduced protein content of the aqueous fractions of model milk systems necessitated the determination of copper in this fraction by difference.

C. Sulfhydryl Group Blocking Agents.

The blocking agents used in whole milk and model systems were added to the milk immediately before heat treatment.

Iodoacetamide, prepared by a method according to Anson (1a) (See Appendix) and N-ethylmaleimide (NEM) were dissolved in distilled water before addition to the raw milk. Raw milk containing 500 µg added copper per liter, (i) 100 ppm NEM or (ii) 100 ppm Iodoacetamide, was subjected to the flash heat treatments employed in experiments with whole and model milk systems. A representative sample of each heat treated milk was then subjected to a nitroprusside test (37) in order to determine the extent of reaction of the sulfhydryl group blocking agents with heat activated sulfhydryl groups.

D. Experimentation.

Experiment 1 and 2. The effect of flash heat treatment on the distribution of residual and added copper in fluid milk.

Morning milk was obtained from a Holstein cow in her fourth month of lactation. This milk was treated with 100 µg Cu/l, flash heated to temperatures ranging from 155 to 190 F (1) and 160 to 200 F (2) in ten degree increments, cooled, and stored 12 hours at 34 F prior to separation.

Experiment 3. The effect of flash heat treatment on the distribution of residual and added copper in fluid milk.

The morning milk obtained from a Holstein cow in her fourth month of lactation was treated with 100 µg Cu/l. The milk was subjected to flash heat treatment to temperatures ranging from 90-200 F in five and ten degree increments, cooled and separated immediately at 270 rcf for 1 hour at 34 F.

Experiment 4. The effect of flash heat treatment on the distribution of residual and added copper in fluid milk.

Morning milk obtained from a Jersey cow in her fourth month of lactation was treated with 200 µg Cu/l. The milk was then flash heated to temperatures ranging from 110 to 200 F in five and ten degree increments, cooled and stored for 18 hours at 34 F prior to separation.

Experiment 5. The effect of heat treatments on the distribution of residual and added copper in fluid milk.

The morning milk of a Holstein cow in her fourth month of lactation was treated with 500 µg Cu/l. This milk was subjected to the following heat treatments:

(A) Flash heating from 140 to 200 F in five and ten degree increments, zero hours of storage at 34 F prior to separation,

(B) Ten minute holding time at each of the flash heat treatments, zero hours of storage at 34 F prior to separation, and (C) Ten minute holding time at each of the flash heat

treatments followed by rapid cooling and storage for 18 hours at 34 F prior to separation.

Experiment 6. The effect of heat treatments on the distribution of residual and added copper in fluid milk.

The morning milk from a Jersey cow in her second week of lactation was treated with 300 µg Cu/l and subjected to the heat treatments as described in the preceding experiment.

Experiment 7. The effect of 10 minute heat treatments on the distribution of copper added to fluid milk before and after heating.

The morning milk from a Jersey cow in her fourth month of lactation, was treated with 400 µg of Cu/l before (B) and following a 10 minute heat treatment (C) at temperatures ranging from 140 to 200 F in five and ten degree increments. A control series (A) of milk containing only residual copper was also subjected to these heat treatments.

Experiment 8. The effect of flash heat treatment on the distribution of two levels of added copper in fluid milk.

The morning milk from a Jersey cow in her first month of lactation was treated with 200 µg (A) and 500 µg (B) Cu/l respectively. This milk was then subjected to flash heat treatments ranging from 150 to 200 F in five and ten degree increments.

Experiment 9. The distribution of added copper in flash heated skimmilk mixed with unheated cream.

The morning milk from a Brown Swiss cow in her fourth month of lactation was divided into two equal portions. One volume was treated with 500 µg Cu/l (A) and flash heated from 160 to 200 F in five and ten degree increments.

The second portion of milk containing only residual copper was delivered in 100 ml aliquots to centrifuge separatory funnels and centrifuged at 270 rcf for 1 hour at 34 F. The skimmilk fractions were combined and treated with 500 µg Cu/l. One hundred ml portions of this skimmilk were flash heated to temperatures from 160 to 200 F, cooled to 80 F and mixed with the unheated cream (B) from the original 100 ml sample of raw milk. This recombined milk was allowed to stand in the centrifuge separatory funnels for 2 hours at 34 F prior to separation at this temperature.

Experiment 10. The effect of washing (36 F) on heat-induced adsorbed copper in the cream fraction of fluid milk.

The morning milk from a Brown Swiss cow in her fourth month of lactation was treated with 500 µg Cu/l. The treated milk was subsequently flash heated to temperatures ranging from 160 to 200 F, cooled, and separated at 34 F. The skimmilk fraction was drawn from the separatory funnel and the copper content determined. The remaining cream fraction was

washed twice with two 100 ml aliquots of redistilled water at 36 F, followed by reseparation in an International centrifuge for 1 hour at 34 F. The washed cream fraction was then analyzed for copper content.

Experiment 11. The effect of washing (80 F) on heat-induced adsorbed copper in the cream fraction of fluid milk.

The preceding experiment was repeated using redistilled water at 80 F to wash to cream fraction.

Experiment 12. The effect of NEM on heat-induced copper migration to the cream fraction of fluid milk.

The morning milk from a Brown Swiss cow in her fourth month of lactation, was treated with 500 μg Cu/l (A). A portion of this milk was treated with 200 ppm N-ethylmaleimide (B). The milks were then flash heated to temperatures ranging from 160 to 200 F, cooled to 80 F and separated at 34 F.

Experiment 13. The effect of NEM on heat-induced copper migration to the cream fraction of fluid milk.

The preceding experiment was repeated with milk from a Jersey cow in her fourth month of lactation. The effect of heat treatment on the distribution of residual copper in the fluid milk was also examined.

Experiment 14. The effect of flash heat treatment on

the distribution of copper in model systems containing micellar casein and supernatant from spun casein.

Model milk systems were prepared from milk dialyzate, washed cream, 500 µg added Cu/l, (A) micellar casein and (B) supernatant from the spun casein. These two model systems were subjected to flash heat treatment of temperatures ranging from 160 to 200 F, cooled, and centrifugally separated at 34 F.

Experiment 15. The effect of flash heat treatment on the distribution of copper in a model system containing 2.5% purified casein.

A model system was prepared using 2.5% purified casein (sodium caseinate), washed cream, milk dialyzate and 500 µg Cu/l. This system and a control system containing no added copper were flash heated to temperatures from 160 to 200 F, cooled and separated at 34 F.

Experiment 16. The effect of flash heat treatment on the distribution of copper in a model system containing Beta-lactoglobulin.

A model system comprised of 0.2% Beta-lactoglobulin (N.B.C., 3X), milk dialyzate, washed cream and 500 µg Cu/l was flash heated to temperatures from 160 to 200 F followed by cooling and separation at 34 F.

A control system comprised of all components but Beta-lactoglobulin was subjected to the same series of flash heat treatments.

Experiment 17. The effect of NEM on heat-induced copper migration in a model milk system containing micellar casein.

A model system prepared from milk dialyzate, washed cream, micellar casein and 500 µg Cu/l was flash heated to temperatures from 160 to 200 F, cooled and separated at 34 F. This procedure was repeated with 200 ppm NEM added to the system prior to flash heat treatment.

Experiment 18. The effect of NEM on the heat-induced migration of copper in a model milk system containing the supernatant from spun casein.

The preceding experiment was repeated with the exception that the supernatant from the spun casein was substituted for micellar casein dispersed in milk dialyzate.

Experiment 19. The effect of Iodoacetamide on heat-induced migration of copper in a model milk system containing 2.5% purified casein.

A model milk system was prepared from milk dialyzate, washed cream, 2.5% purified casein and 500 µg Cu/l. A series of control samples (B) containing no sulfhydryl group blocking agent and samples to which had been added 200 ppm

Iodoacetamide (A) were flash heated to temperatures from 160 to 200 F and subsequently cooled and separated at 34 F.

Experiment 20. The effect of NEM on the heat-induced migration of copper in a model milk system devoid of skimmilk proteins.

A control model system (B) composed only of milk dialyzate, washed cream and 500 µg Cu/l was flash heated to temperatures from 160 to 200 F, cooled and separated at 34 F. This model system containing 200 ppm NEM (B) was subjected to the flash heat treatments employed by the control series.

RESULTS

A. Sensitivity of Copper Determination.

The carbamate method applied to 326 freeze-dried fractions of heated milk (Experiments 1-8, inclusive) resulted in an average copper recovery of 97.9% with a standard deviation of ± 2.0%. Calculation of the recovery of added copper from approximately 1800 samples showed little deviation from this degree of accuracy. The innovation of the freeze-drying step in the carbamate procedure permitted multiple experimentation on a common sample of milk.

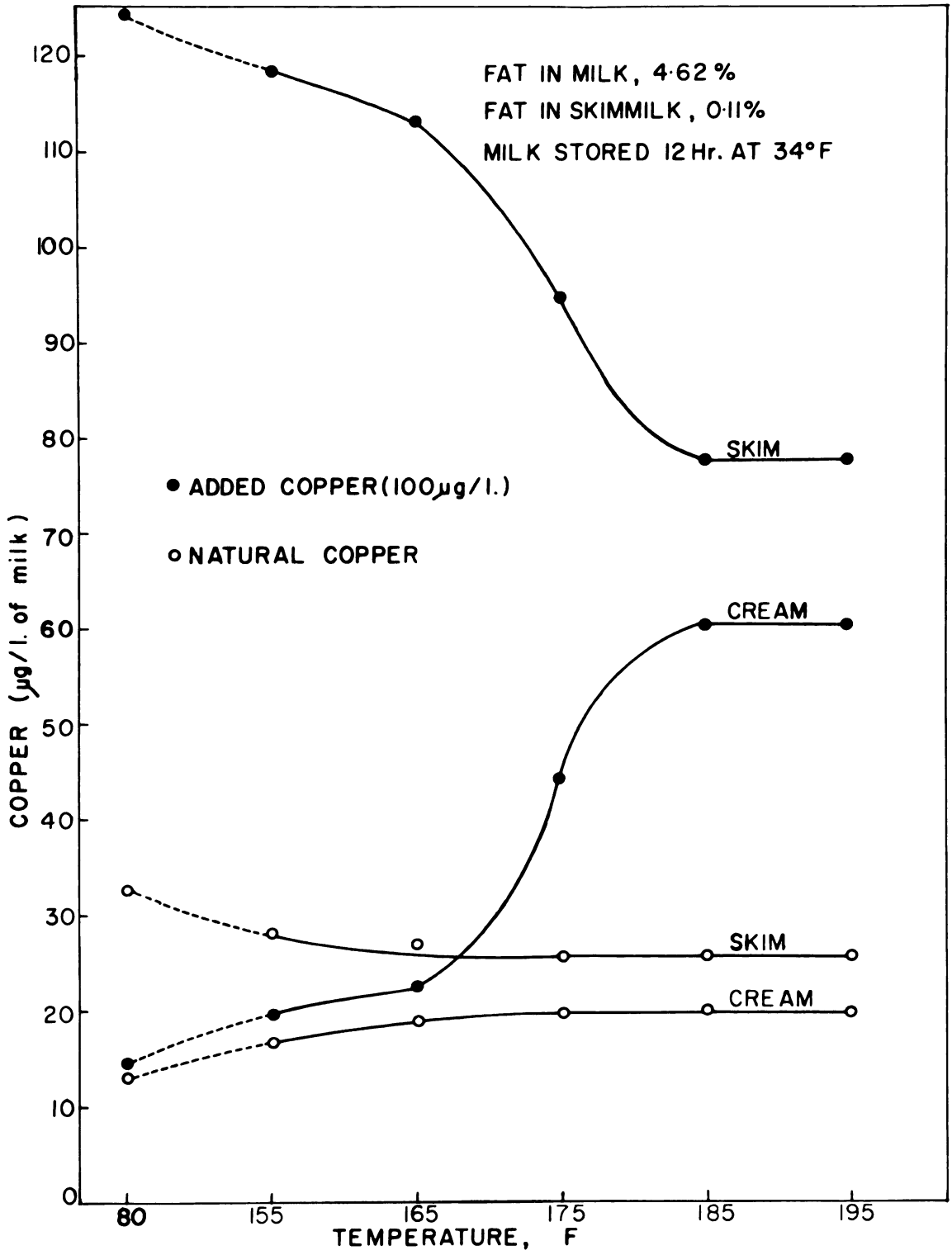

FIGURE 1. The effect of flash heat treatment on the distribution of residual and added copper in fluid milk.
(Flash heat treatment, 155-195 F)

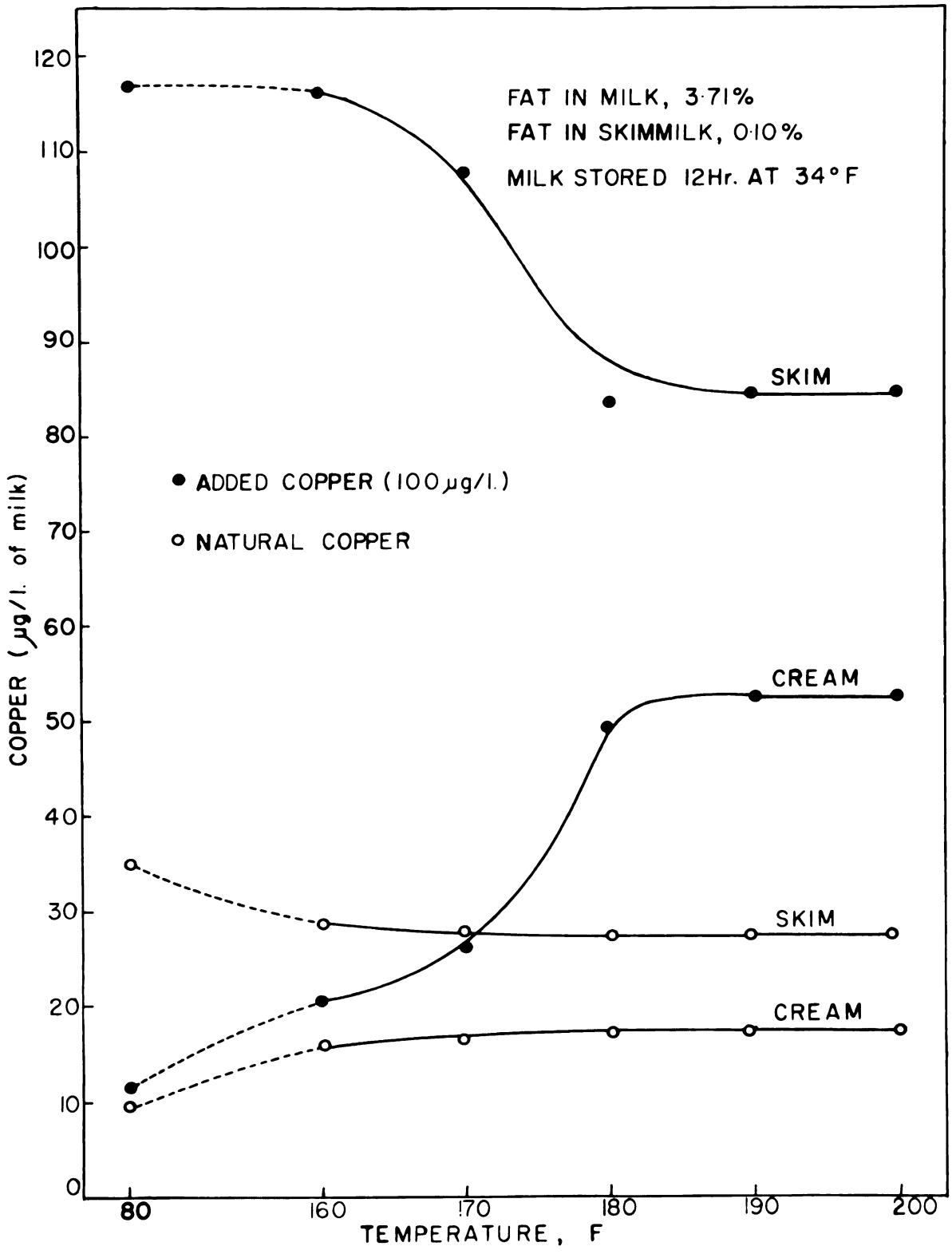

FIGURE 2. The effect of flash heat treatment on the distribution of residual and added copper in fluid milk.
(Flash heat treatment, 160-200 F)

B. Experimentation. (Note: The number on the graph corresponds to order of the performed experiment).

Experiment Nos. 1 and 2.

A momentary heat treatment of Holstein milk (residual copper, 45 µg/l; added copper, 100 µg/l) to 155 F induced a detectable migration of residual and added copper from the skimmilk to the cream fraction. Maximum and constant migration of copper to the cream was noted in milk flash heated from 180 to 200 F. The copper content in the cream fraction of milk flash heated in this temperature range increased by as much as 420 per cent. Storage of the unheated milk for 12 hours at 34 F resulted in a 2-3 per cent increase in the copper content of the cream fraction. (Figure 1 and 2).

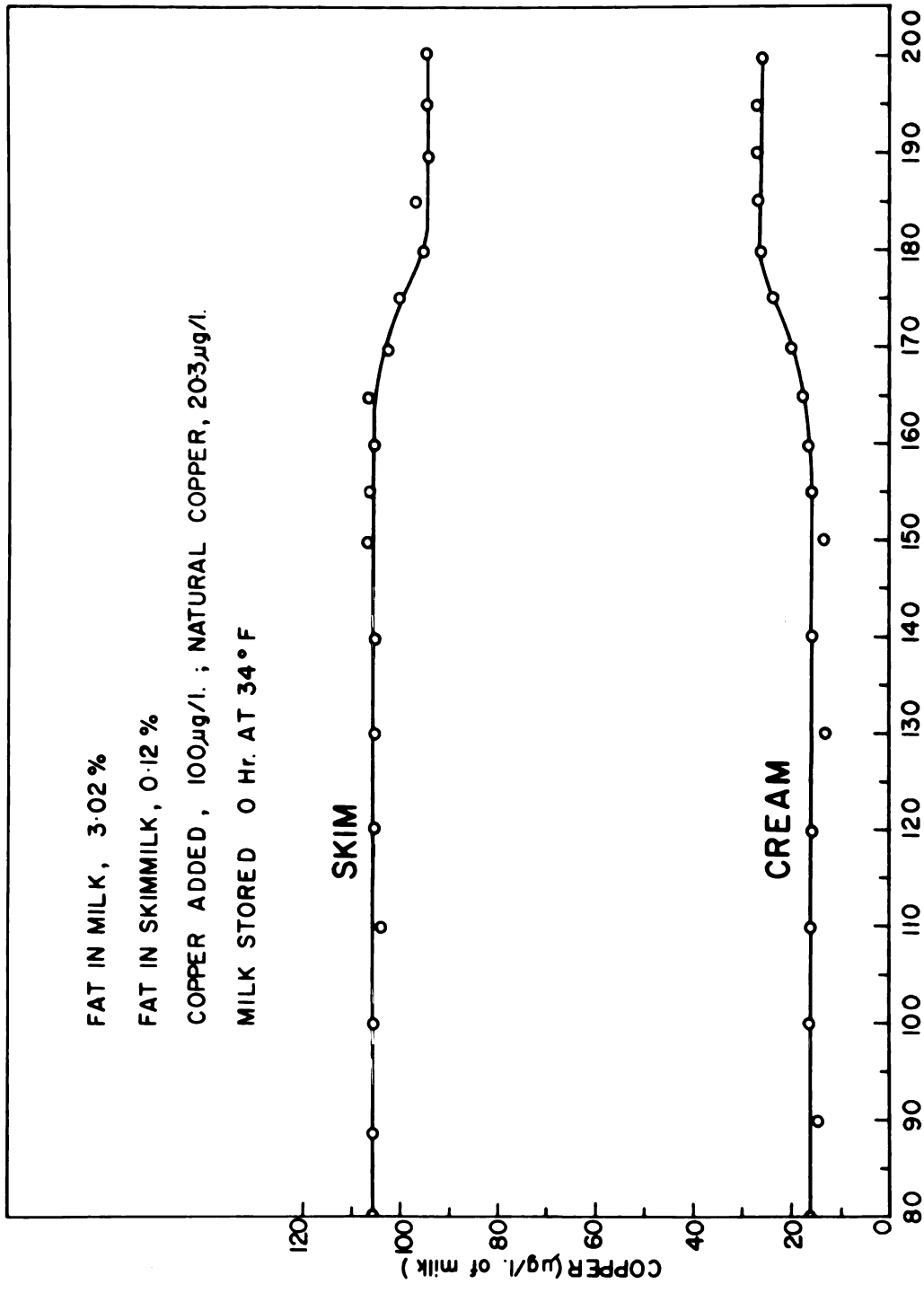

FIGURE 3. The effect of flash heat treatment on the distribution of residual and added copper in fluid milk. (Flash heat treatment, 90-200 F)

Experiment No. 3.

Holstein milk, flash heated to temperatures from 90 to 200 F with no subsequent storage at 34 F, exhibited initial migration of copper to the cream following momentary heating at 160 F. Maximum and constant migration of copper was again noted in the cream fraction of milk subjected to flash heat treatment of 180 to 200 F. (Figure 3).

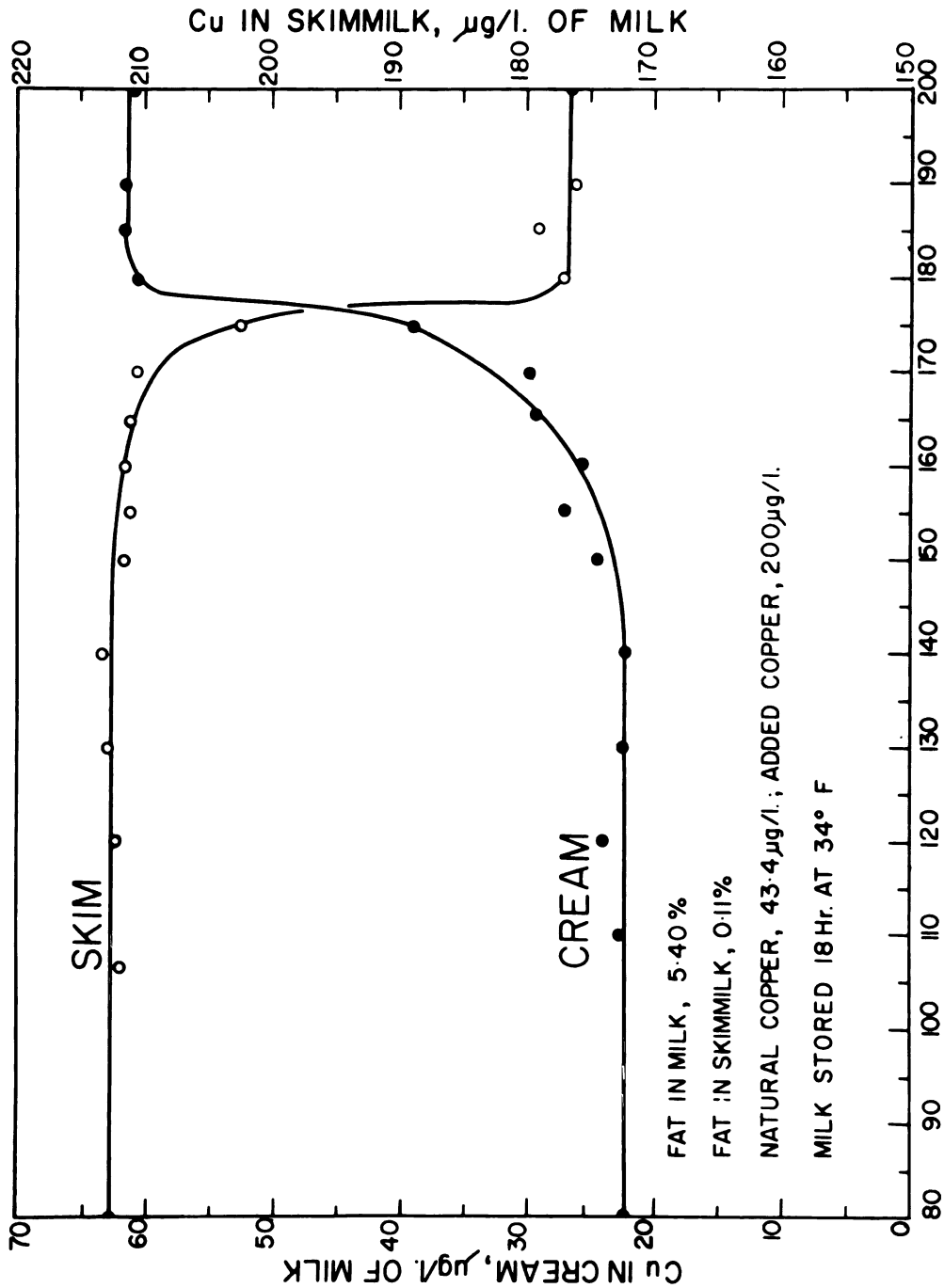

FIGURE 4. The effect of flash heat treatment on the distribution of residual and added copper in fluid milk. (Total copper, 243µg/l)

Experiment No. 4.

In this experiment, the total copper content of Jersey milk was raised to 243 µg Cu/l by the addition of 200 µg of added copper per liter. Flash heat treatment of the milk to temperatures greater than 140 F induced migration of copper from the skimmilk to the cream fraction. Storage of the milk for 18 hours at 34 F prior to separation did not appear to alter the temperature range at which maximum and constant migration of copper to the fat fraction occurred; maximum and constant copper adsorption by the cream phase was again observed in the milk flash heated from 180 to 200 F. The total copper content of the cream increased by 270 per cent as a result of heat treatment in this temperature range. (Figure 4).

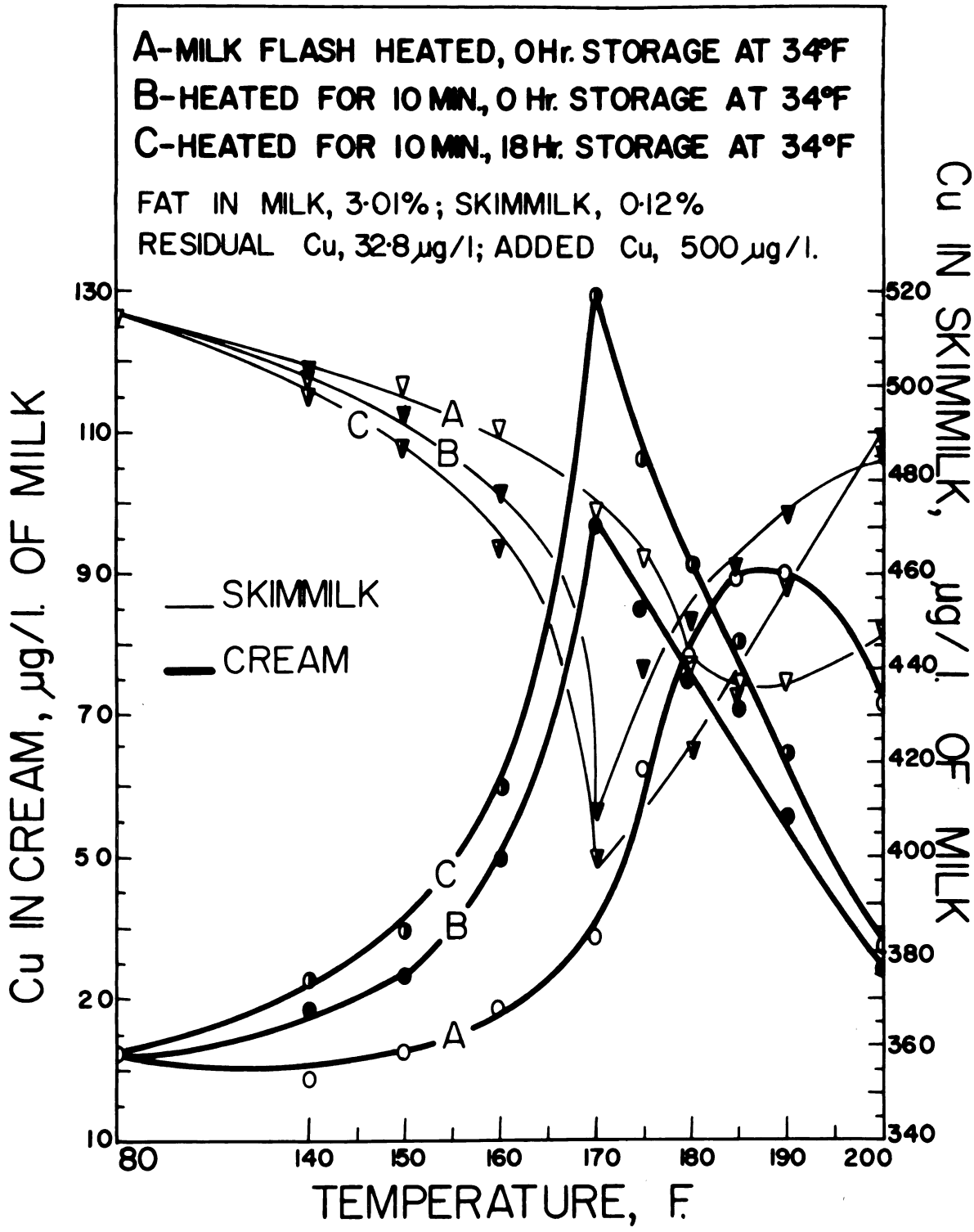

FIGURE 5. The effect of heat treatments on the distribution of residual and added copper in fluid milk. (Added copper, 500µg/l)

Experiment No. 5.

Figure 5 illustrates the effects of three separate heat treatments of Holstein milk containing 532 µg Cu/l. Flash heating of this milk with no storage at 34 F prior to separation (A) resulted in maximum migration of copper to the cream in milk heated to 185-190 F. The total copper content of the cream fraction increased by 410 per cent in this temperature range. When the milk was subjected to a 10 minute holding period at each of the flash heat temperatures (B), maximum adsorption of copper by the cream was detected in milk held at 170 F. Storage of the milk for 18 hours prior to separation (C) resulted in an increase in the extent of copper migration to the cream at the 170 F heat treatment. At this temperature, the total copper content of the cream increased by 580 per cent.

In each of the three heat treatments of the milk, desorption of copper from the cream was noted as a result of heating the milk to temperatures greater than that temperature inducing maximum adsorption of copper by the cream.

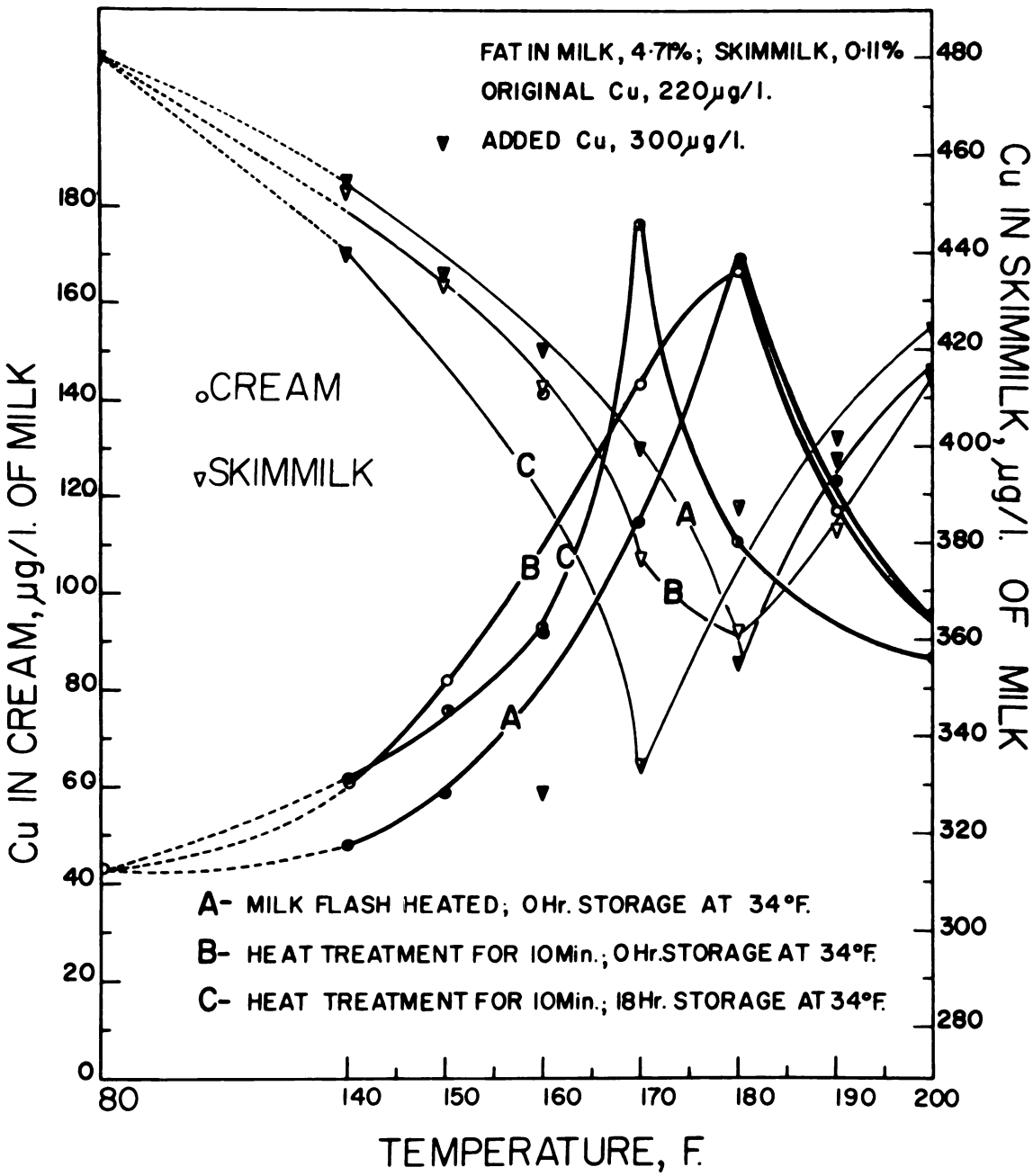

FIGURE 6. The effect of heat treatments on the distribution of residual and added copper in fluid milk. (Added copper, 300µg/l)

Experiment No. 6.

The preceding experiment was repeated with milk from a Jersey cow in her second week of lactation. The cream fraction demonstrated maximum adsorption of copper following flash heating (A) or 10 minute holding at 180 F (B). When the milk was stored 18 hours at 34 F prior to separation (C), maximum migration of copper to the cream was observed in milk heated at 170 F for 10 minutes, as previously noted in Experiment No. 6. In addition, the greatest change in the extent of heat-induced copper migration occurred in the milk subjected to heat treatments of 160 to 170 F. (Figure 5 and 6).

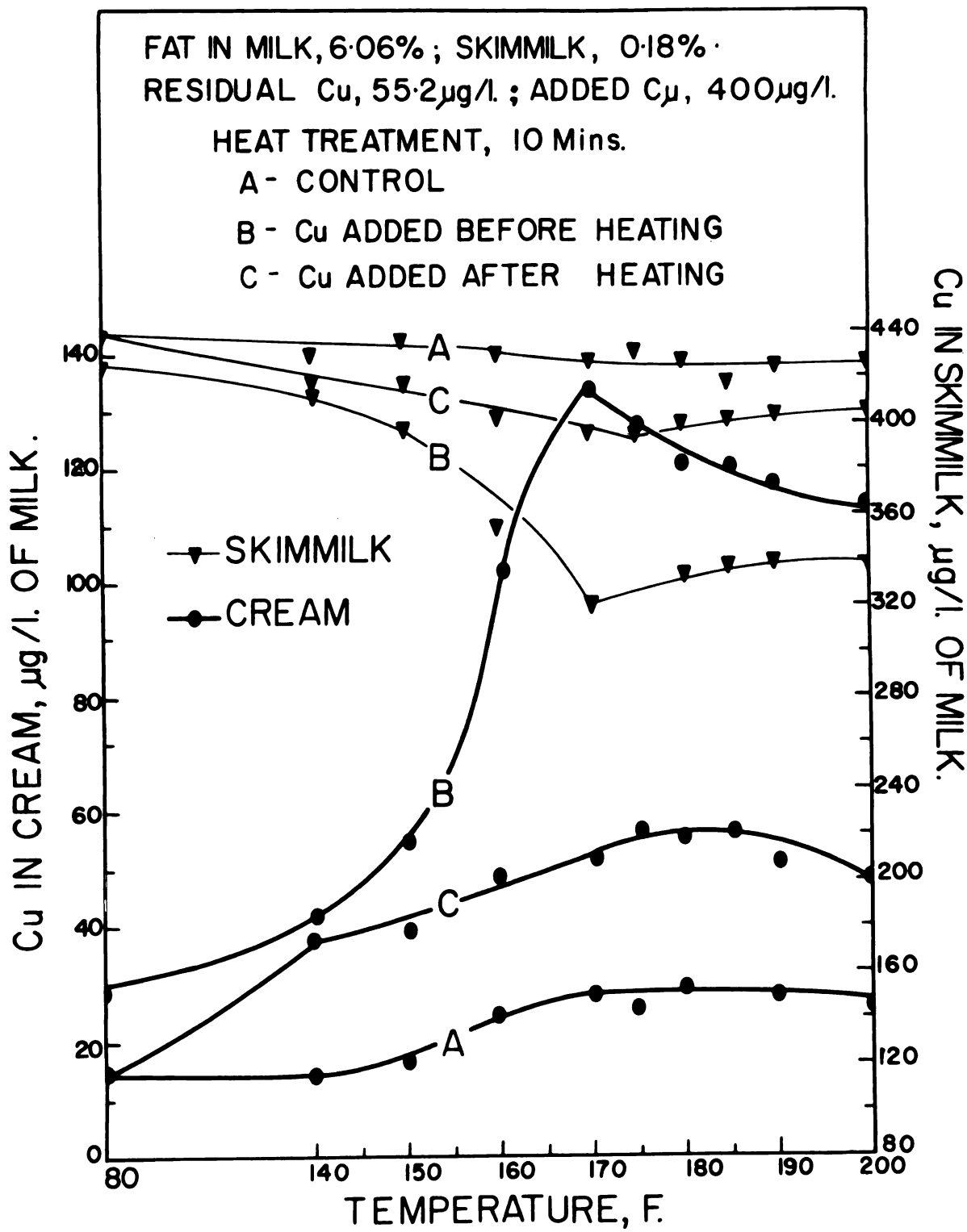

FIGURE 7. The effect of 10-minute heat treatments on the distribution of copper added to fluid milk before and after heating.

Experiment No. 7.

Figure 7 illustrates the effects of heating Jersey milk for 10 minutes at temperatures ranging from 140 to 200 F. The control sample (A) containing only residual copper demonstrated maximum and constant adsorption of copper by the cream phase following 10 minute heat treatments at 180 F and above. Treating the milk with 400 µg Cu/l prior to heating (B) resulted in a maximum copper adsorption peak in cream phase at 170 F. On the other hand, when the same amount of copper was added to the milk following heat treatment (C), maximum adsorption of copper by the cream phase, but to a lesser extent than treatment B, was noted in the range of 175 ot 185 F. The desorption phenomenon was again evident in the cream fractions of copper treated milk following heating at temperatures greater than 170 F. (Curve B).

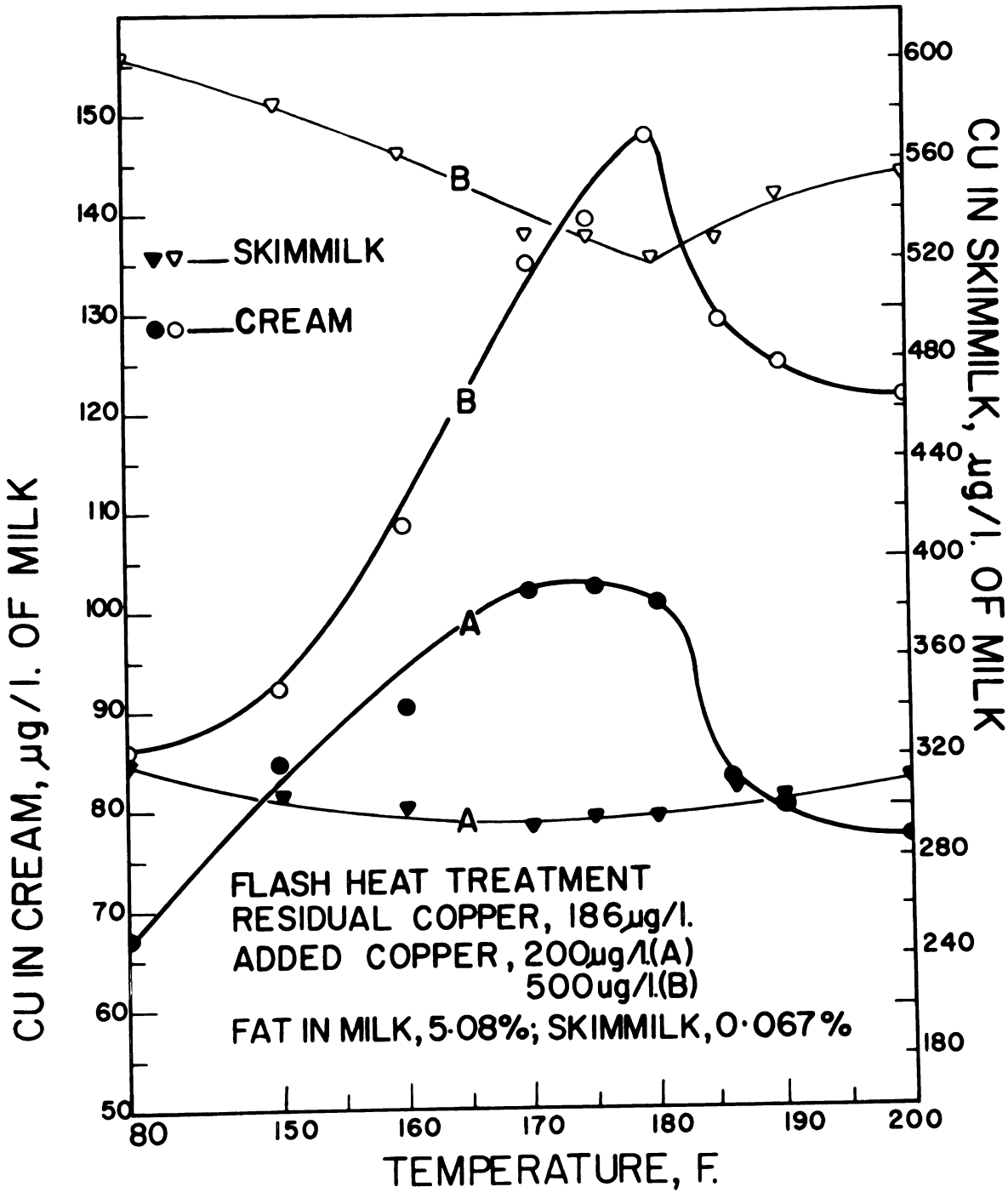

FIGURE 8. The effect of flash heat treatment on the distribution of two levels of added copper in fluid milk.

Experiment No. 8.

Milk from a Jersey cow in her first month of lactation was treated with 200 and 500 µg Cu/l respectively. At the lower level of added copper, curves labeled A on Figure 8, maximum adsorption of copper by the cream occurred following flash heat treatments of 170 to 180 F. The subsequent decrease in copper content of the cream at higher temperatures indicated heat-induced desorption of copper. The same milk containing the higher level of added copper, curves labeled B on the graph, exhibited maximum adsorption by the cream phase at 180 F. Once again, heating the milk to temperatures in excess of 180 F resulted in a lower copper content in the fat fraction.

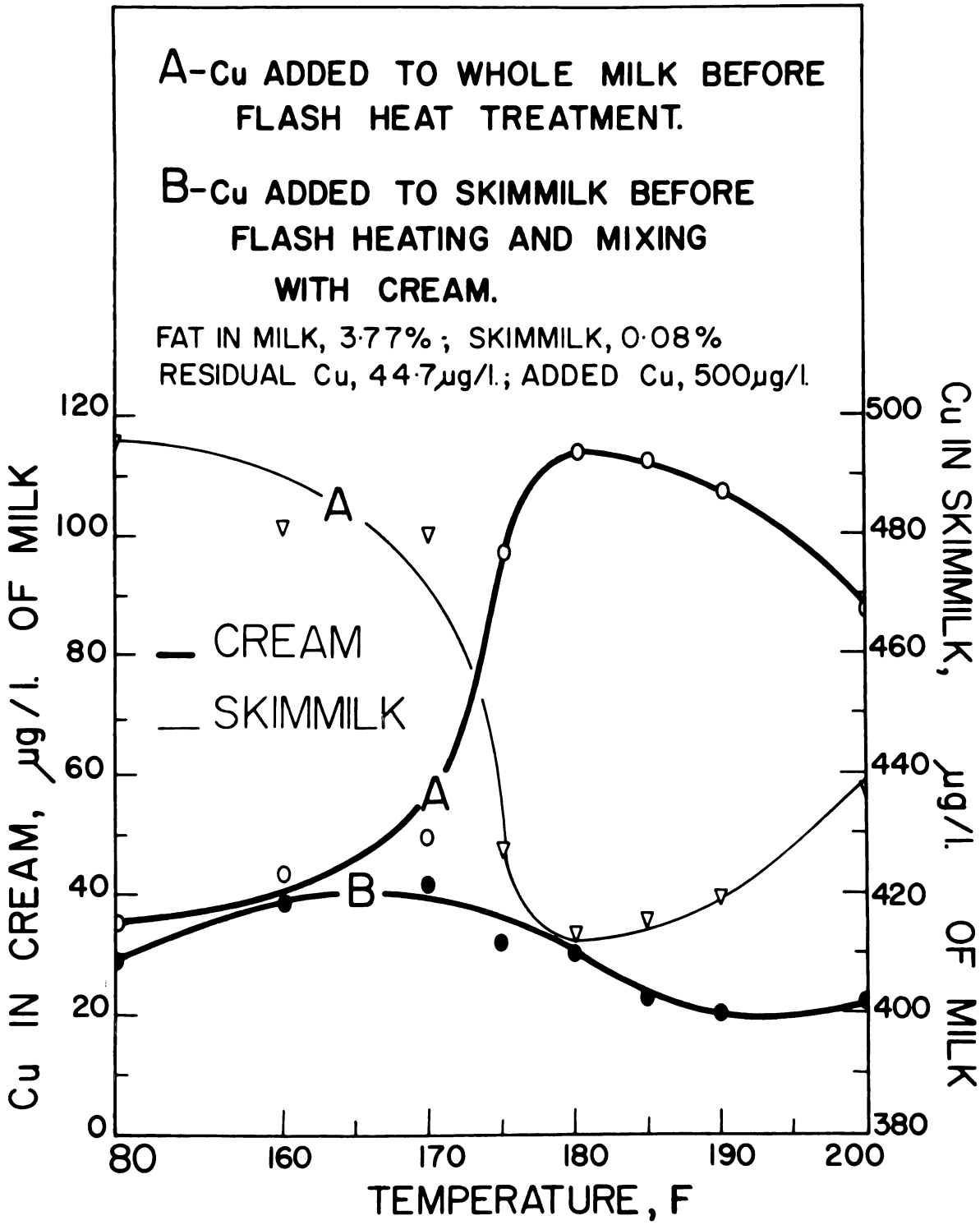

FIGURE 9. The distribution of added copper in flash heated skimmilk mixed with unheated cream.

Experiment No. 9.

Raw skimmilk treated with 500 µg Cu/l was flash heated from 160 to 200 F and mixed with unheated cream. A slight but maximum copper adsorption was detected in cream mixed with skimmilk heated to 170 F (Figure 9, Curve B). The control samples (A) containing 545 µg Cu/l exhibited the characteristic copper adsorption peak in the cream of milk flash heated 180 F followed by the desorption phenomenon at higher temperatures.

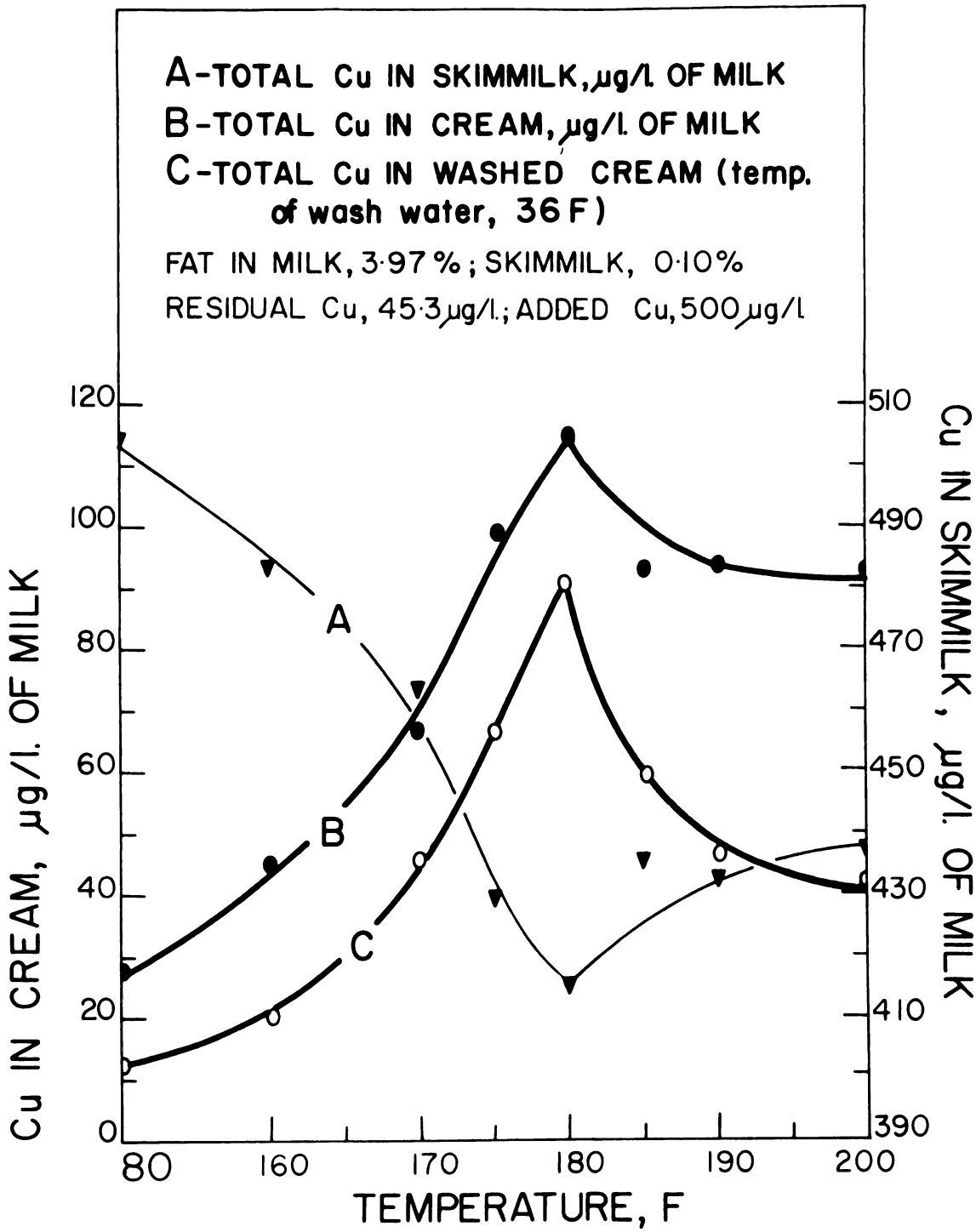

FIGURE 10. The effect of washing (36 F) on heat-induced adsorbed copper in the cream fraction of fluid milk.

Experiment No. 10.

The copper adsorption-desorption phenomenon was again observed in Brown Swiss milk treated with 500 µg Cu/l and exposed to the flash heat treatments (B). The total copper content of the cream fraction increased by 420 per cent and attained a maximum value in milk heated to 180 F. Washing the cream fractions with redistilled water at 36 F (C) removed a relatively constant quantity of adsorbed copper from the cream of milk flash heated from 160 to 180 F; however, washing the cream of milk flash heated to 180 to 200 F removed as much as 43 per cent of the total copper content. Copper was most tenaciously adsorbed in the washed cream of milk flash heated to 180 F. (Figure 10).

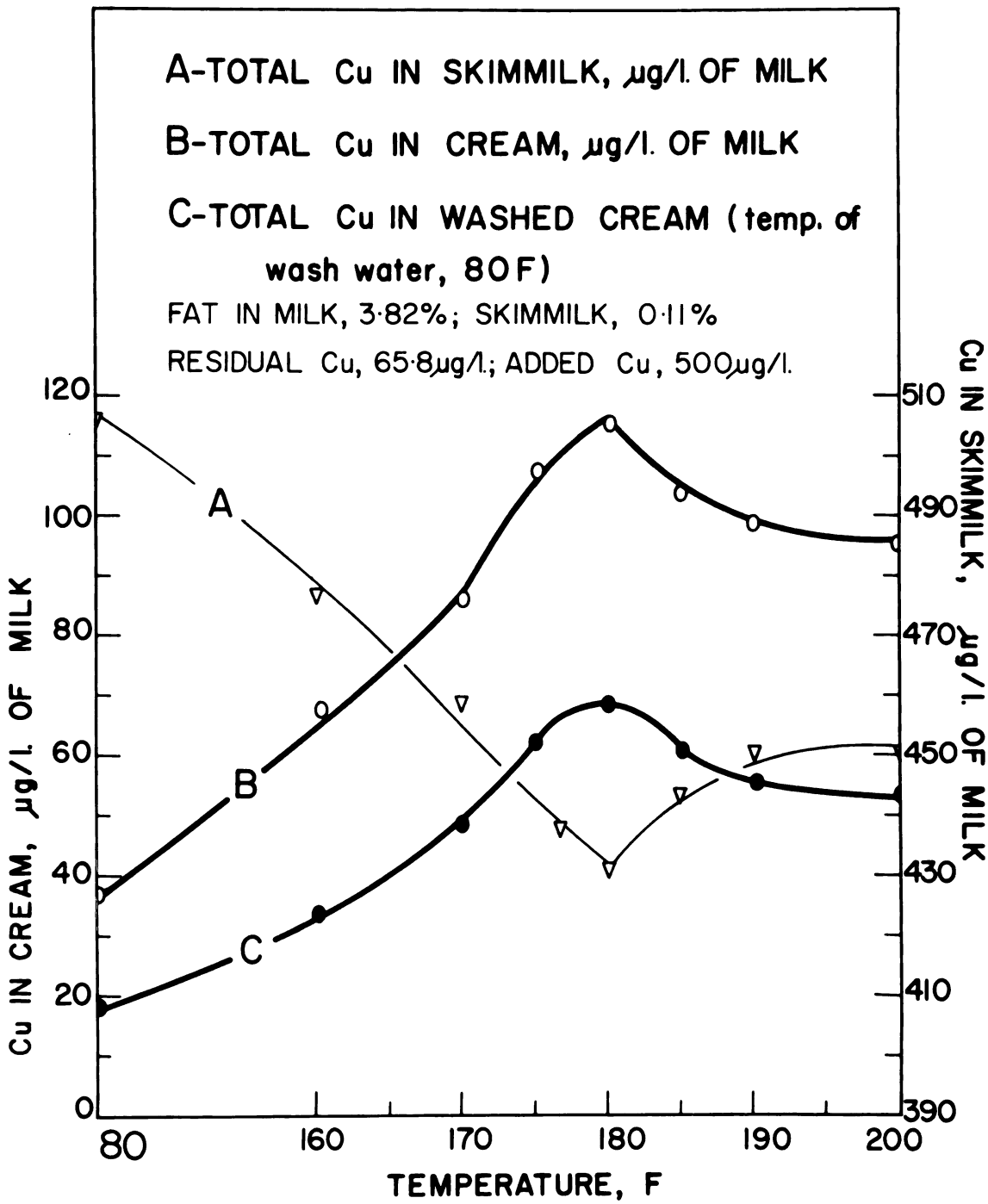

FIGURE 11. The effect of washing (80 F) on heat-induced adsorbed copper in the cream fraction of fluid milk.

Experiment No. 11.

The preceding experiment was repeated using redistilled wash water at 80 F (Figure 11). Washing the cream with water at a higher temperature failed to alter the characteristic pattern of heat-induced copper adsorption in the cream fraction; however, the loss of total copper in the cream as a result of washing increased throughout the series of heated milks. As much as 53 per cent of the total copper was washed from the cream of milk heated to 180 F (C).

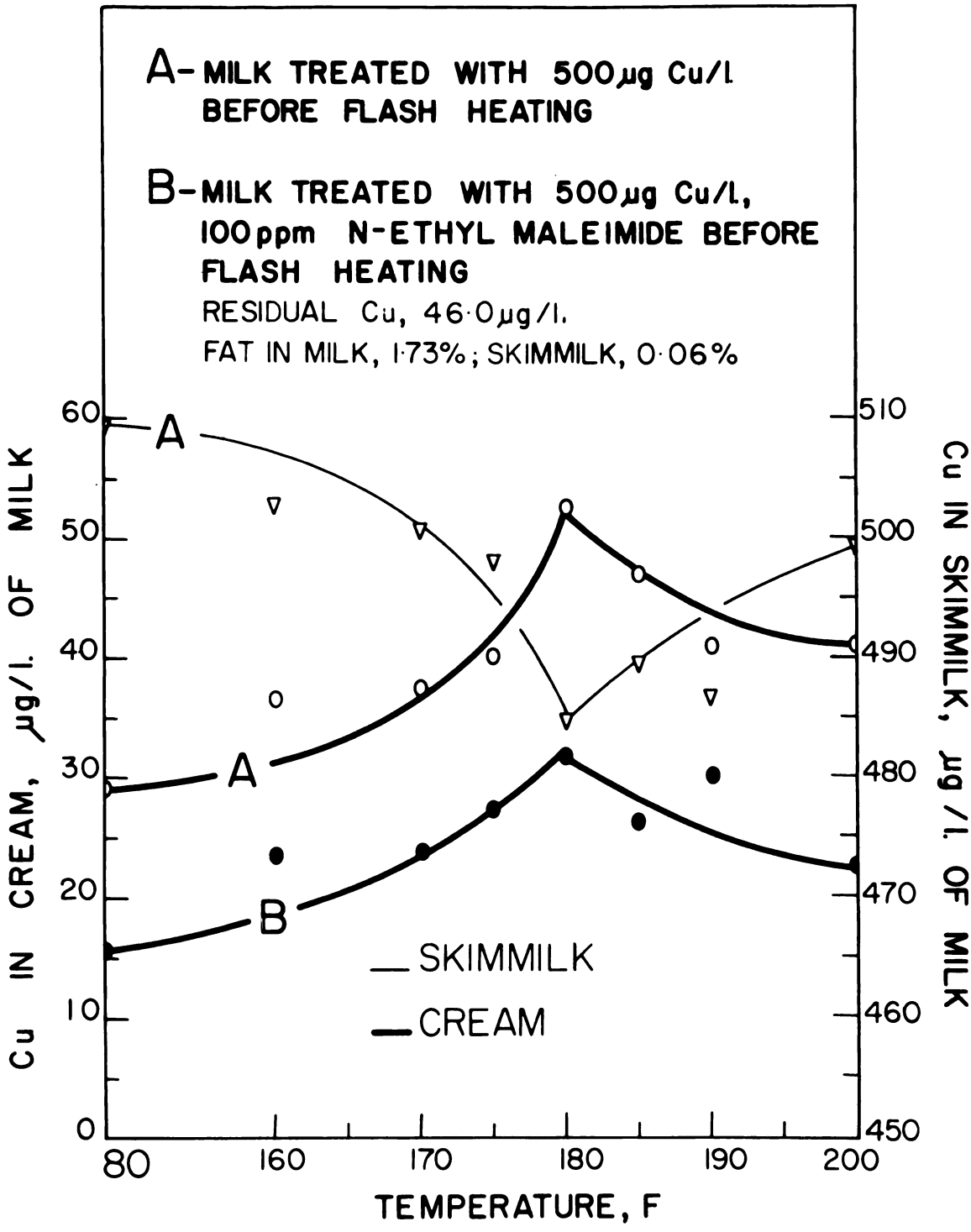

FIGURE 12. The effect of NEM on heat-induced copper migration to the cream fraction of fluid milk.

Experiment No. 12.

Figure 12 illustrates the effects of a sulfhydryl group blocking agent on heat-induced copper migration to the cream. The control sample (A) containing no NEM exhibited the copper adsorption peak in the cream fraction of milk flash heated to 180 F. When the milk was treated with 200 ppm NEM prior to flash heating at 180 F, the extent of migration of copper to the cream was reduced by approximately 40 per cent.

The fat content of the Brown Swiss employed in this experiment was abnormally low as a result of inclusion of frozen silage in the cow's ration.

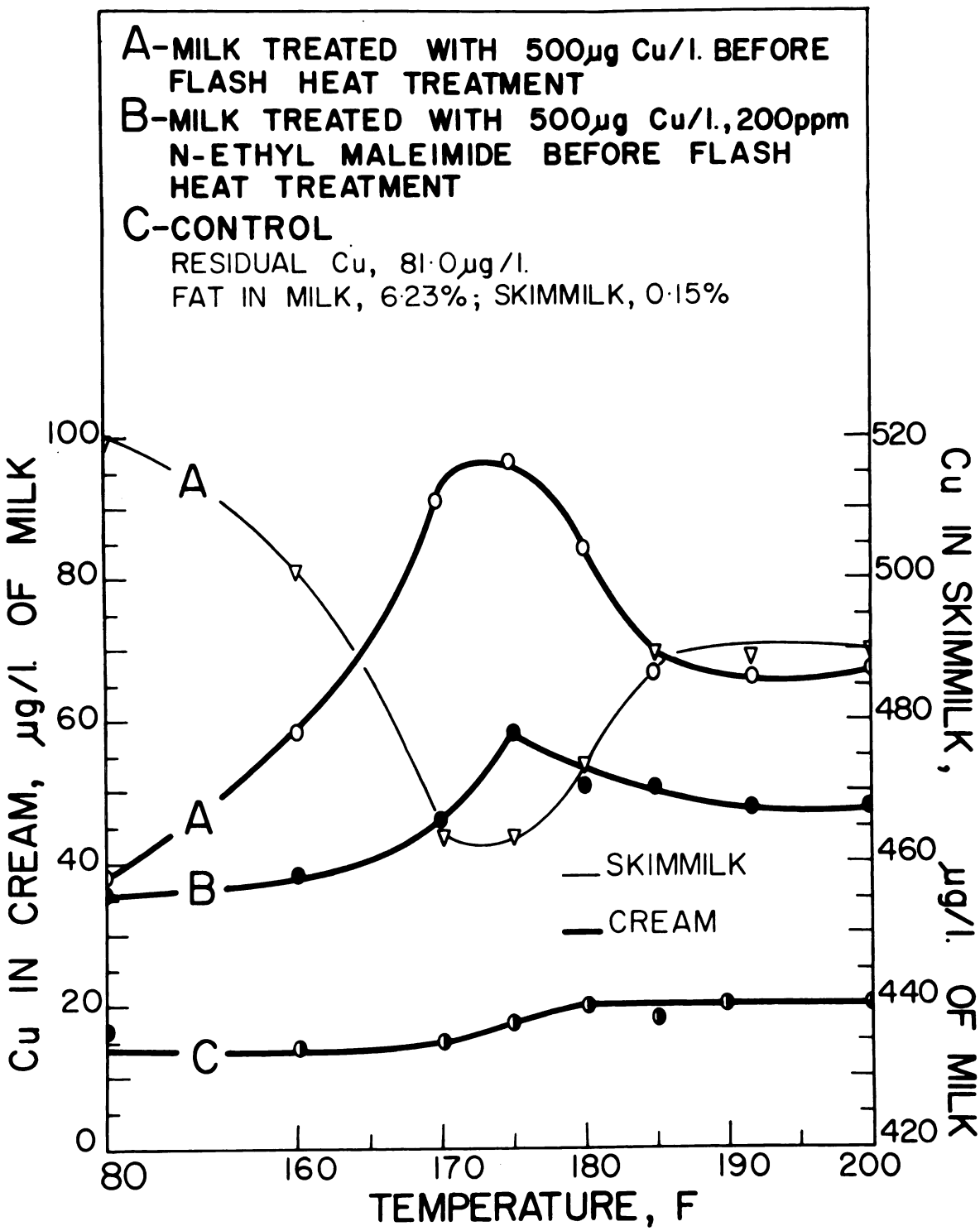

FIGURE 13. The effect of NEM on heat-induced copper migration to the cream fraction of fluid milk.

Experiment No. 13.

The preceding experiment was repeated with Jersey milk. This milk, treated with 500 µg Cu/l, (Figure 13, Curve A) demonstrated maximum copper adsorption in the cream phase at 175 F rather than 180 F as previously noted. When the milk was treated with 200 ppm NEM prior to heating (B), the extent of migration of copper to the cream as a result of flash heating at 175 F was reduced by 65 per cent. The control sample (C) containing only residual copper exhibited the characteristic constant migration of copper to the cream in milk flash heated from 180 to 200 F.

An aliquot of the heated milk containing NEM was sampled following each of the flash heat treatments and subjected to the nitroprusside test. In each case, a negative nitroprusside test indicated that heat activated sulfhydryl groups were blocked by NEM. The same milk treated with 200 ppm iodoacetamide prior to flash heating from 160 to 200 F also yielded a negative nitroprusside test.

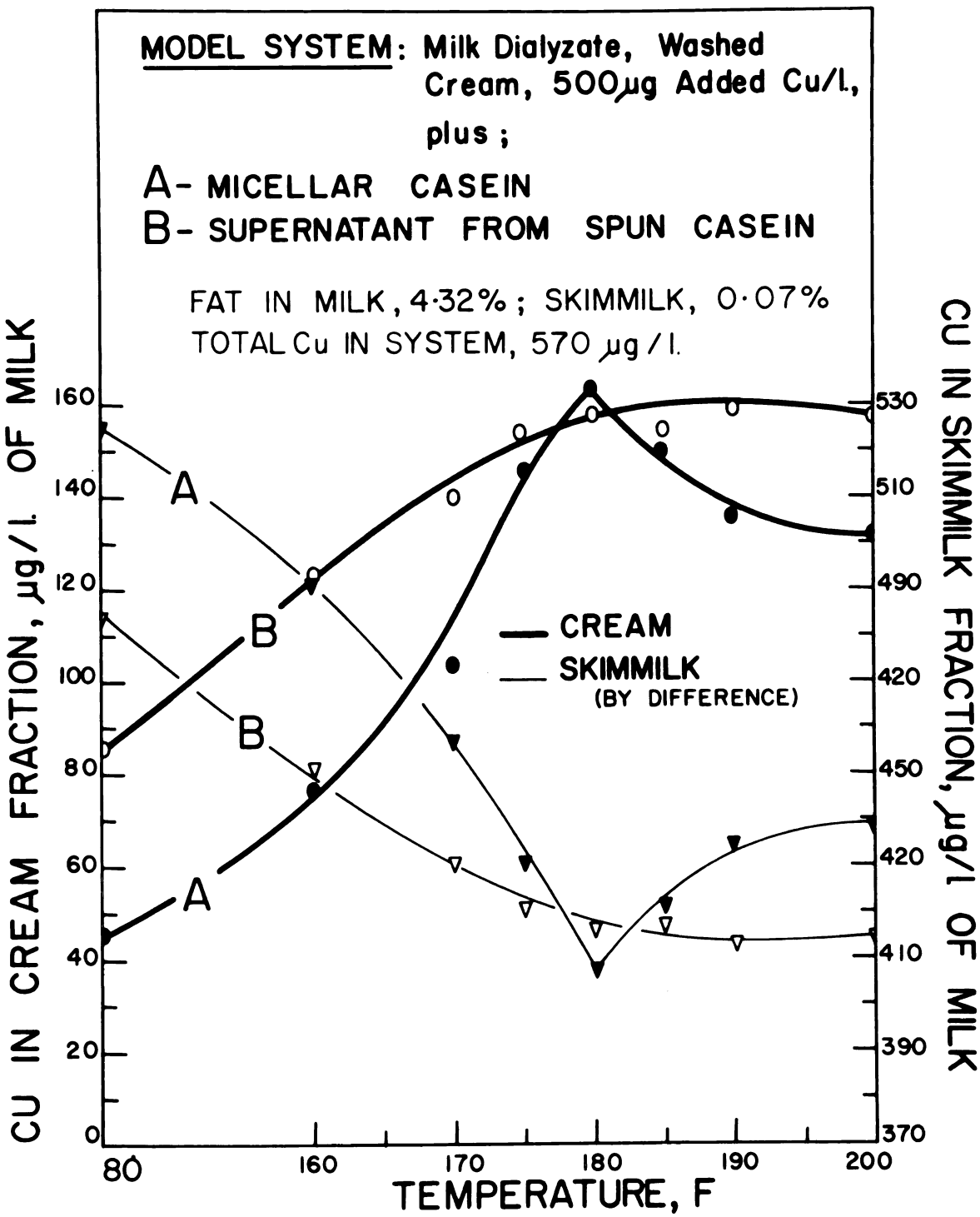

FIGURE 14. The effect of flash heat treatment on the distribution of copper in model systems containing micellar casein and supernatant from spun casein.

Experiment No. 14.

Curves labeled A on Figure 14 illustrate the effect of flash heat treatment on a model milk system of milk dialyzate, washed cream, micellar casein obtained at 41,000 rcf for 6 hours, and 500 µg added Cu/l. Maximum adsorption of copper by the fat fraction was observed when the system was subjected to momentary heating at 180 F; desorption of copper from the cream phase occurred at higher flash heat temperatures.

System B, in which the milk dialyzate dispersion of micellar casein was replaced by the supernatant of centrifugally separated casein, failed to demonstrate the copper adsorption peak in the cream fraction in this system, the copper content of the cream attained a maximum and constant value following flash heating from 180 to 200 F.

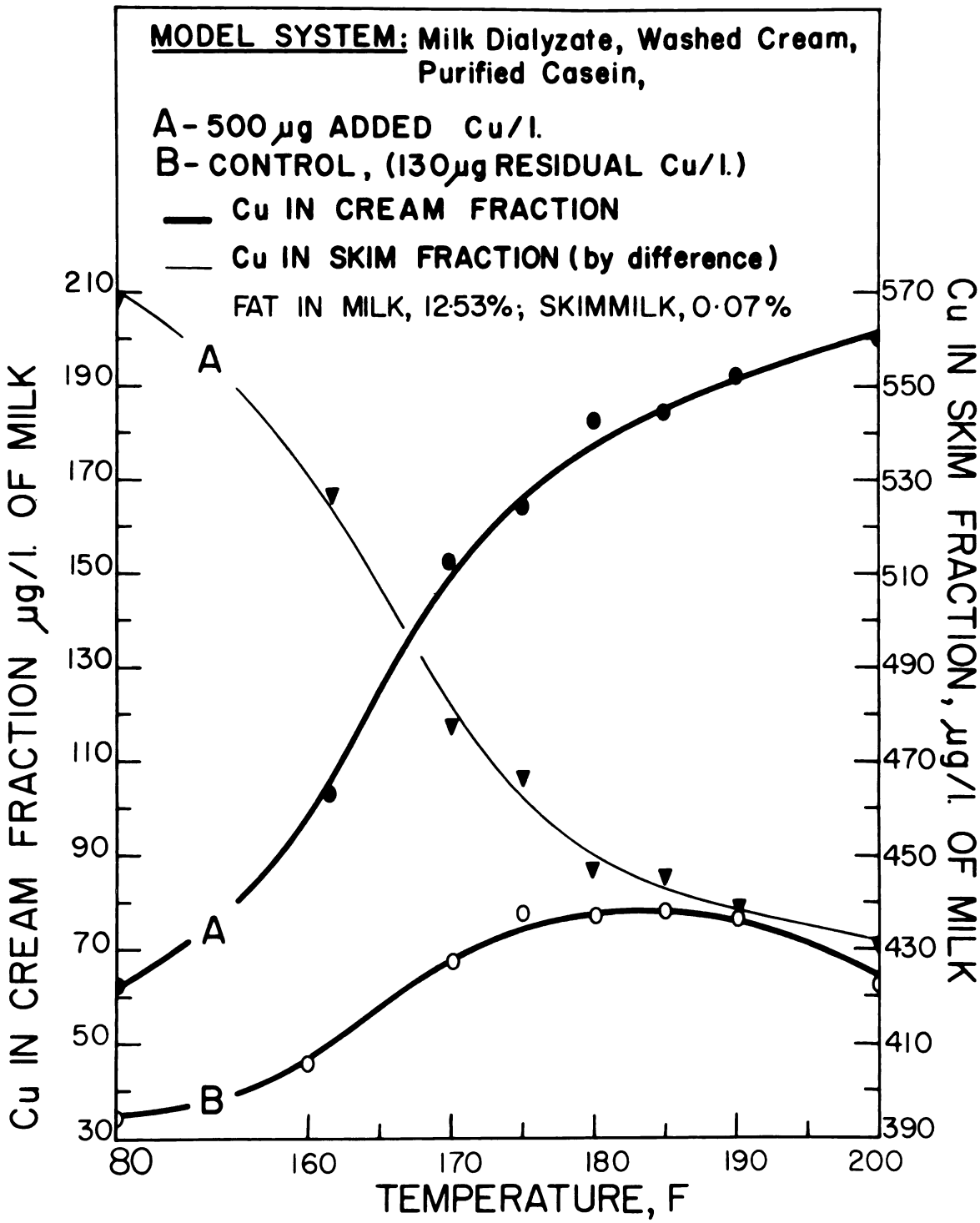

FIGURE 15. The effect of flash heat treatment on the distribution of copper in a model system containing 2.5% purified casein.

Experiment No. 15.

The extent of heat-induced copper adsorption increased with temperature in the model system comprised of milk dialyzate, washed cream, sodium caseinate (2.5%) and 500 ug added Cu/l (Figure 15, Curves A). The control model system (B) containing only residual copper exhibited maximum and constant migration of copper to the cream at flash heat temperatures of 175 to 180 F. Heating this system to 200 F resulted in a slight but detectable loss of total copper content in the cream fraction.

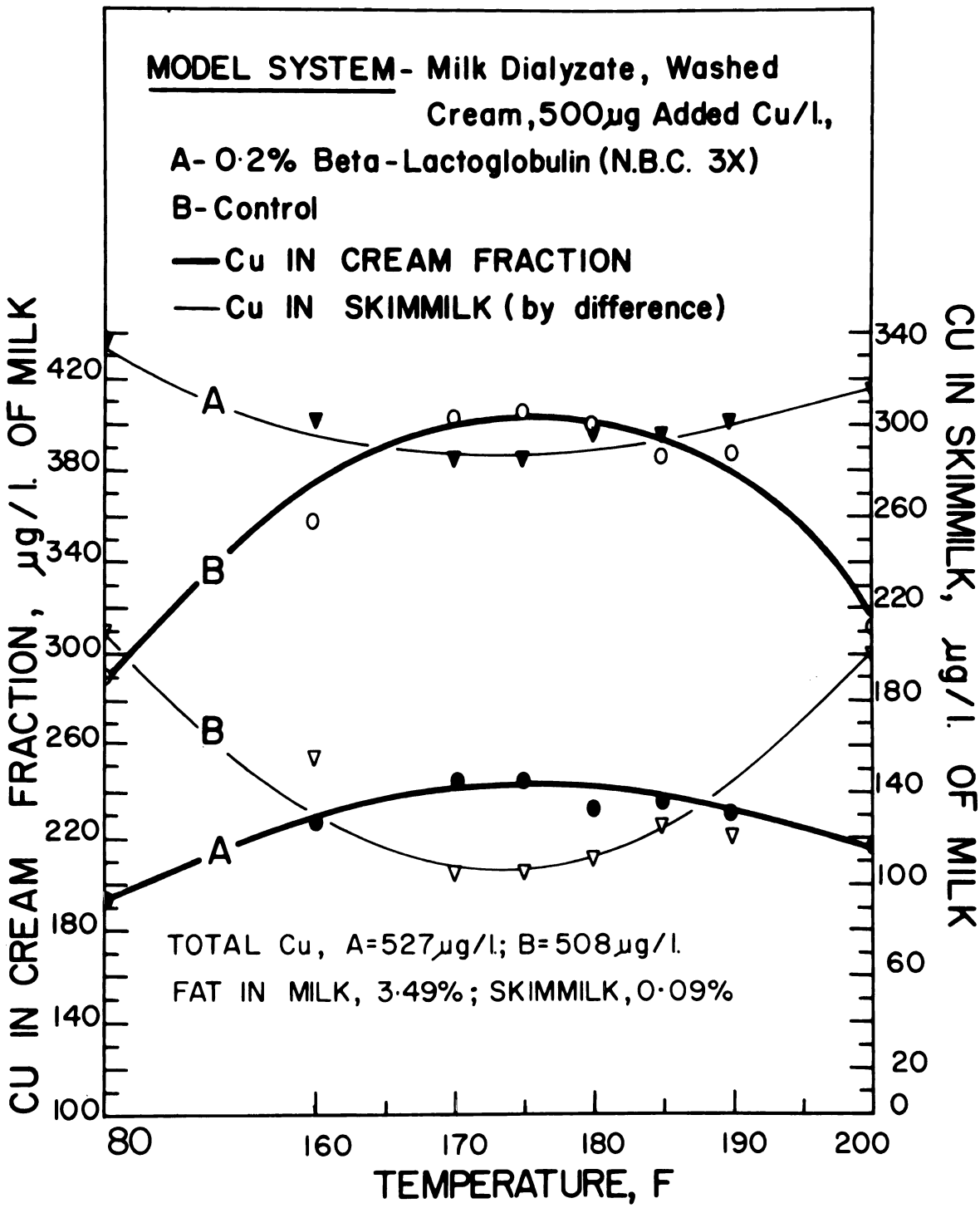

FIGURE 16. The effect of flash heat treatment on the distribution of copper in a model system containing beta-lactoglobulin.

Experiment No. 16.

Figure 16 illustrates the effects of flash heat treatments on the distribution of copper in a model milk system of milk dialyzate, washed cream and 500 µg added Cu/l in the presence and absence of beta-lactoglobulin (0.2%). Heating the system containing this whey protein to temperatures of 170 to 180 F induced maximum migration of copper to the cream phase. As much as 48 per cent of the total copper in the system appeared in the cream fraction as a result of flash heating in this temperature range. (Curve A).

The control system (B) containing no beta-lactoglobulin also demonstrated maximum copper adsorption by the cream fraction following flash heating from 170 to 180 F. It is interesting to note that approximately 80 per cent of the total copper in the system was recovered from the cream fraction of samples heated to these temperatures. Furthermore, as much as 56 per cent of the added copper migrated immediately to the cream fraction upon addition of copper to the system.

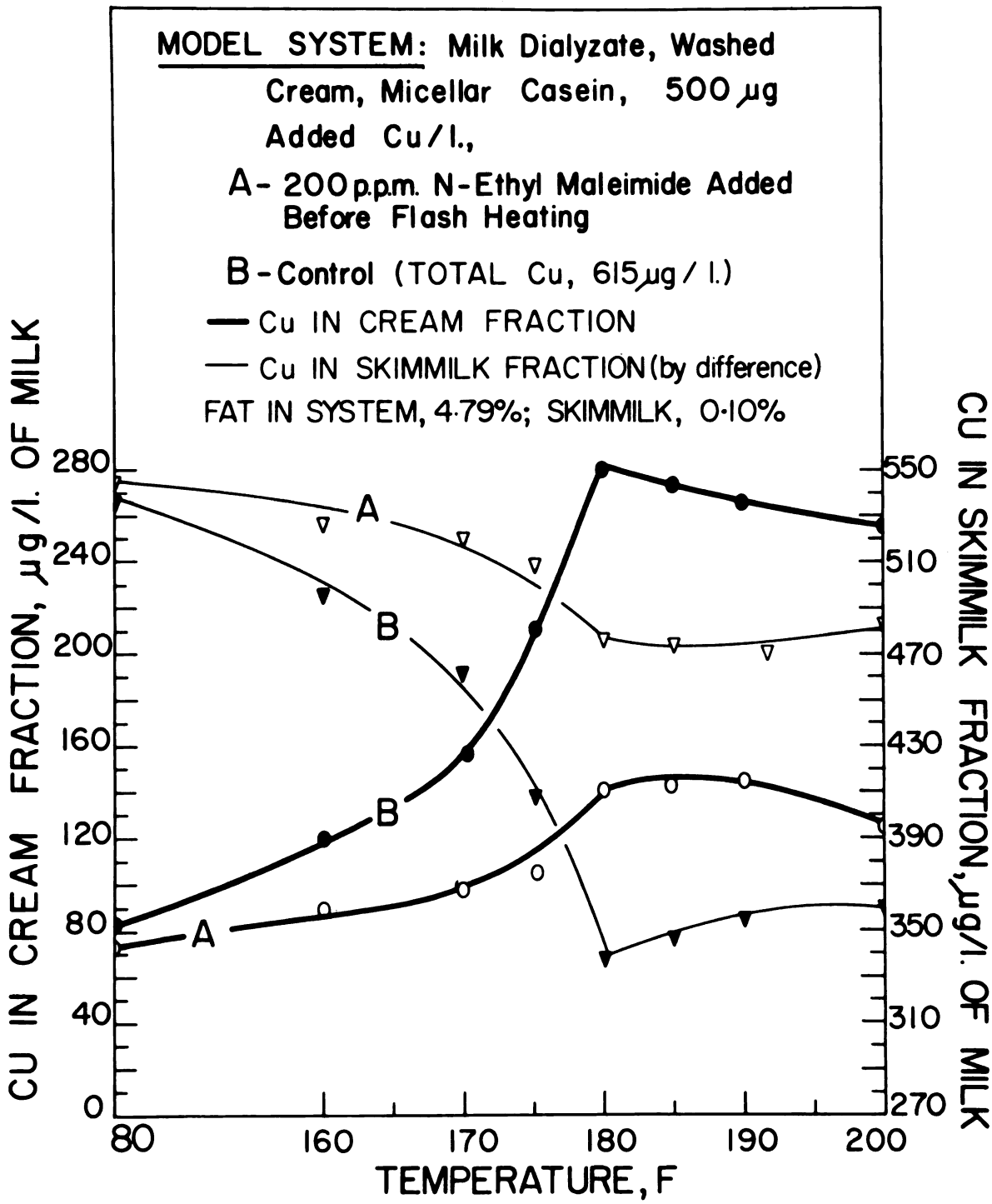

FIGURE 17. The effect of NEM on heat-induced copper migration in a model milk system containing micellar casein.

Experiment No. 17.

Flash heat treatment of a model system containing a milk dialyzate dispersion of micellar casein resulted in copper adsorption in the cream fraction below 180 F with subsequent desorption of copper from the cream fraction at higher temperatures (Figure 17, Curves B). When this system was treated with 200 ppm NEM prior to flash heating, the extent of migration of copper to the cream following flash heating to 180 F was reduced by 65 per cent (Curves A). Once again, the heat-induced copper adsorption-desorption phenomenon in the cream was evident.

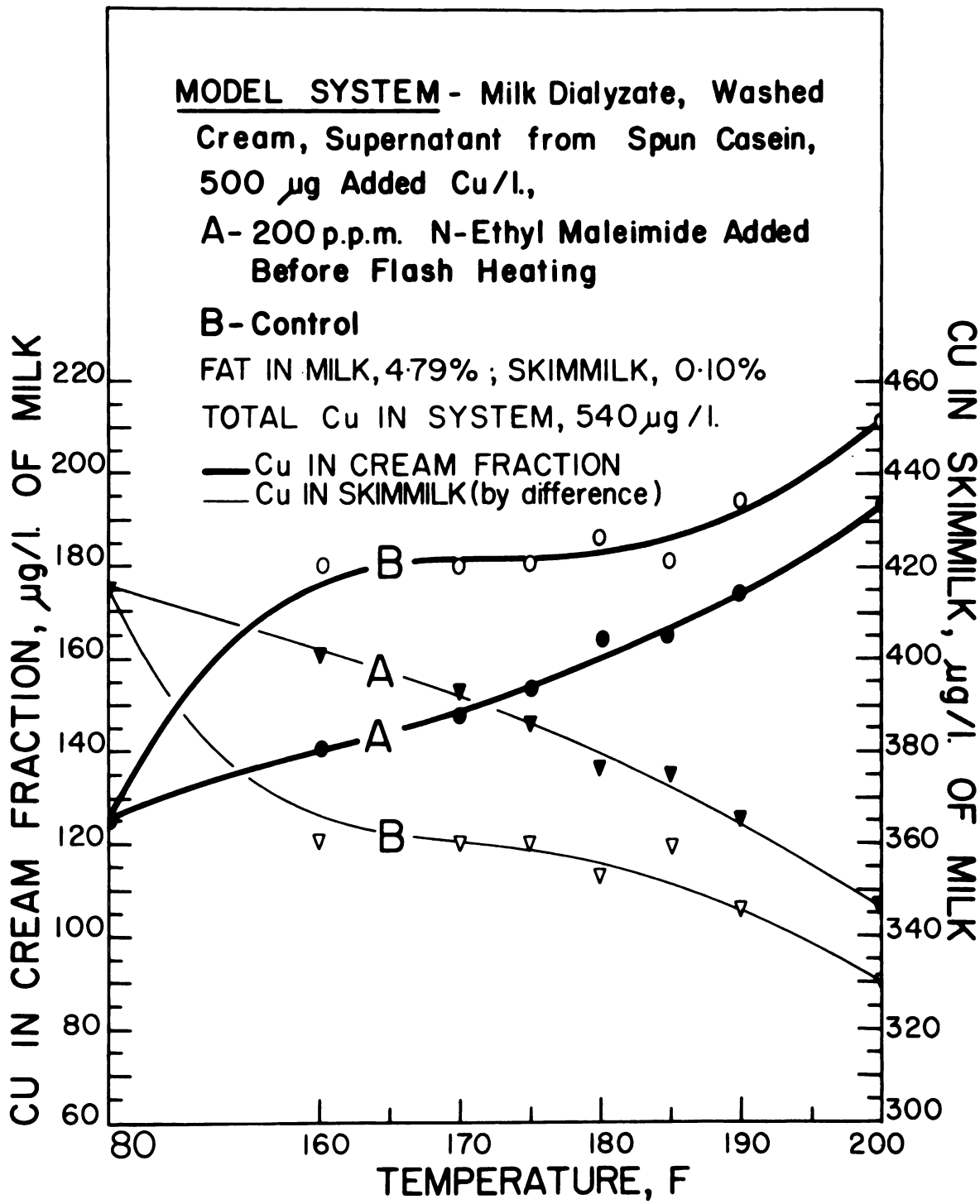

FIGURE 18. The effect of NEM on the heat-induced migration of copper in a model milk system containing the supernatatn from spun casein.

Experiment No. 18.

Figure 18 illustrates the effects of NEM on heat-induced migration of copper to the cream fraction of a model system containing the supernatant from the spun casein used in the preceding experiment. The control model system (B) containing no sulfhydryl group blocking agent failed to exhibit a copper adsorption peak in the cream at a flash heat treatment of 180 F. In this system the copper content of the cream increased with the temperature of flash heating. When 200 ppm NEM was added to the system prior to flash heating, the extent of heat-induced copper migration to the cream was notably reduced at all flash heat temperatures.

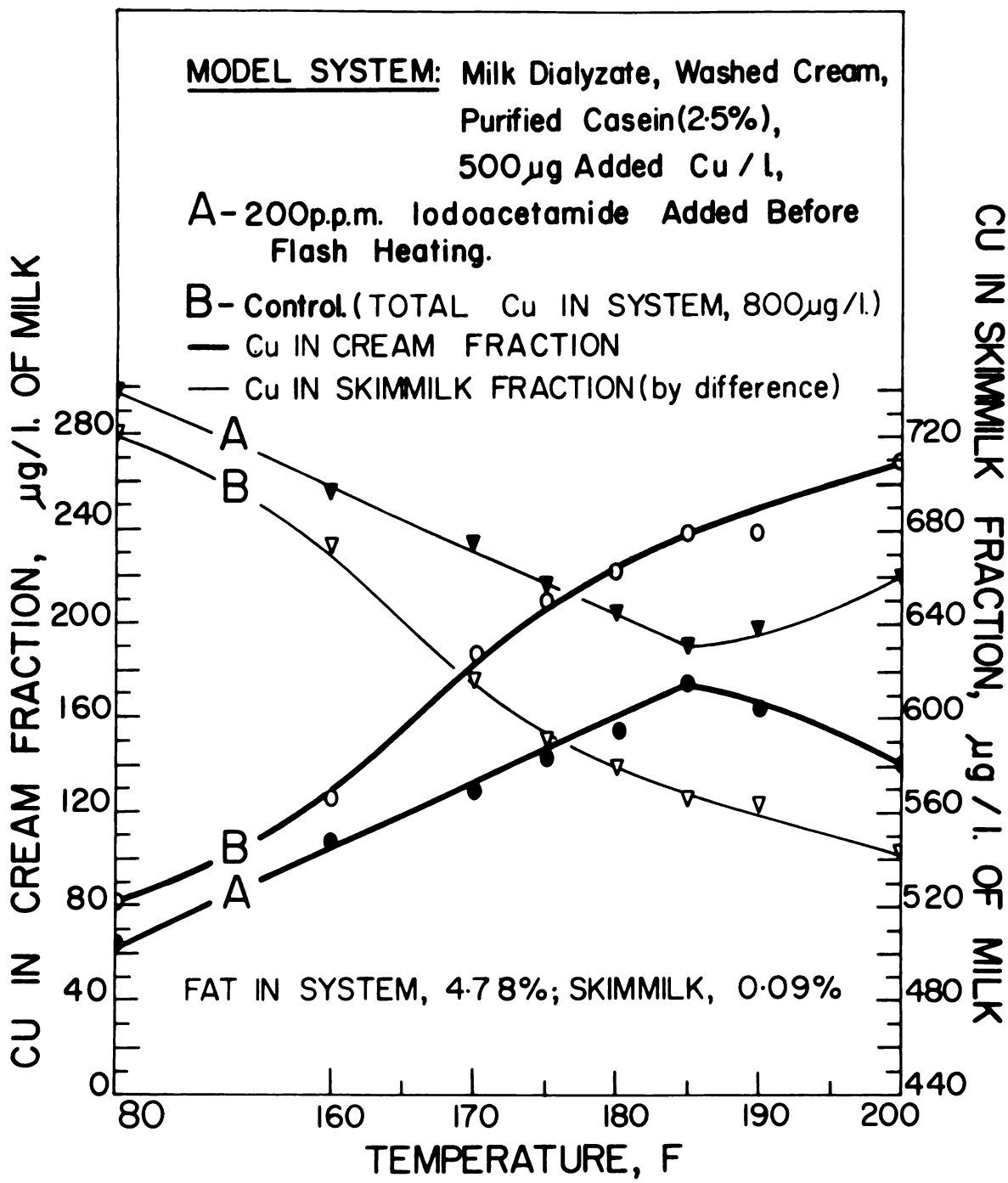

FIGURE 19. The effect of Iodoacetamide on heat-induced migration of copper in a model system containing 2.5% purified casein.

Experiment No. 19.

This experiment was performed to determine whether the addition of iodoacetamide would partially inhibit heat-induced copper migration in a model system containing sodium caseinate. In the control system (B) containing no sulfhydryl group blocking agent, the copper content of the cream fraction increased with temperature as previously reported (Experiment 15). However, when the system was treated with 200 ppm Iodoacetamide prior to heat treatment (A), the extent of heat-induced copper migration to the cream diminished at all flash heat temperatures and the characteristic copper adsorption maximum appeared in the cream at 180 F. The migration of copper to the cream was reduced by 43 per cent in the presence of iodoacetamide at this flash heating to 180 F.

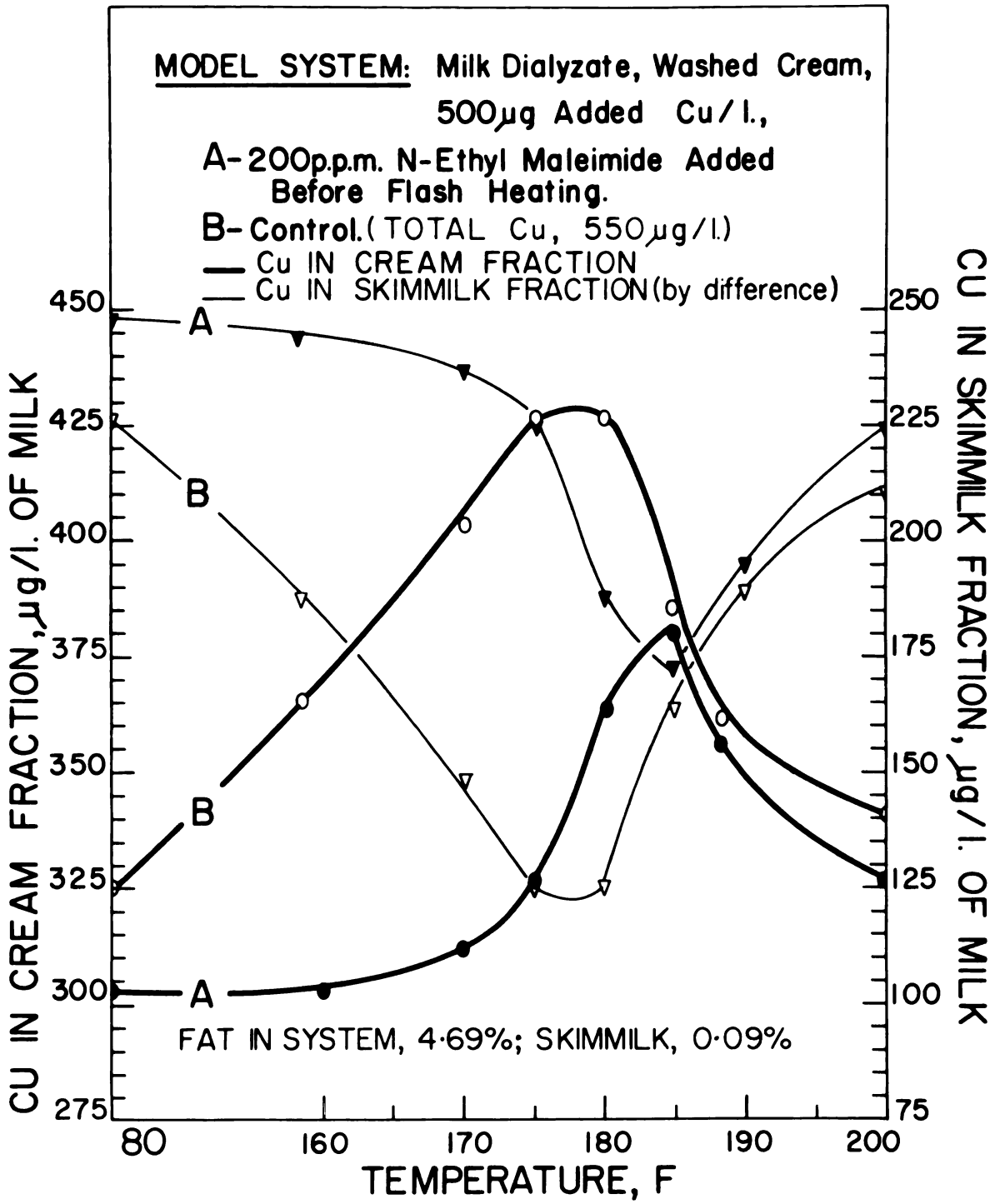

FIGURE 20. The effect of NEM on the heat-induced migration of copper in a model system devoid of skimmilk proteins.

Experiment No. 20.

Flash heat treatment of a model system comprised only of milk dialyzate, washed cream and added copper, (Figure 20, Curves B) induced maximum migration of copper to the cream in the range of 175 to 180 F as reported in an earlier experiment (Experiment 16).

The presence of the sulfhydryl group blocking agent (A), NEM, not only reduced the adsorption of copper by the cream in the unheated system but also reduced the extent of copper migration to the cream by 75 per cent at the flash heat temperature of 175 F. This system demonstrates maximum copper adsorption by the cream at 185 F.

In the control system (B) devoid of skimmilk proteins and sulfhydryl group blocking agent, 45 per cent of the added copper migrated to the cream prior to heat treatment and 75 per cent migrated to the cream as a result of flash heating at 175 F.

DISCUSSION

Raw milk sampled from individual Holstein, Jersey and Brown Swiss cows in their third and fourth month of lactation contained residual copper ranging from 20 to 80 μg Cu/l (Figures 1, 2, 3, 4, 5, 7, 9, 10, 11, 12, and 13). The residual copper content of fresh milk was much higher (186 and 220 μg Cu/l) during the month following parturition (Figures 6 and 8). This observation is in agreement with the findings of earlier investigators (43).

Copper added to fresh raw milk was preferentially bound to the skimmilk proteins, as reported by previous workers (30, 40, 54, 55).

The observation that as little as 2-3 per cent of the added copper migrates to the cream fraction (Figures 1 and 2) agrees with data published by King et al. (40). The affinity of the skimmilk proteins for added copper was substantiated by data obtained from experiments with model milk systems. In the absence of the skimmilk proteins, as much as 75 per cent of the added copper migrated to cream fraction of the unheated model milk system (Figures 16 and 20). No definite statement can be made regarding the distribution of natural copper in raw milk. The data obtained did suggest that natural copper was primarily adsorbed at the fat globule interface; however, the tremendous copper-binding surface

area presented by the small fat globules in the skimmilk (0.1 - 0.2% fat) may have distorted this observation.

The results of experiments involving fluid milk indicated that residual and added copper migrated from the skimmilk to the cream fraction as a result of heat treatment. Milk having a total copper content in the normal range of 40 to 240 µg Cu/l exhibited maximum and constant migration of copper to the cream following flash heating (Figures 1, 2, 3, 4, and 13) or 10 minute holding periods at temperatures of 180 to 200 F (Figure 7). The initial heat-induced migration of copper in milk having a normal copper content was detected at flash heat temperatures in excess of 140 F.

An increase of the total copper content of the milk to that level known to catalyze lipid autoxidation, i.e. 500 - 700 µg Cu/l, resulted in maximum migration of copper to the cream in milk flash heated to approximately 180 F (Figures 5, 6, 8, 9, 10, 11, 12, and 13) or following a 10 minute holding at 170 F (Figures 5 and 7). The author suggests that the difference in occurrence of maximum copper adsorption in the cream fraction may be explained by different rates of reaction at the two temperatures, and that the same adsorption phenomenon is occurring in both heat treatments. On the other hand, the flash heat temperature producing maximum migration of copper to the cream may be characteristic of milk produced by an individual cow. Heating fluid milk systems having a

total copper content in excess of 500 µg/l induced desorption of copper from the cream fraction at flash heat temperatures greater than 180 F (Figures 6, 8, 9, 10, 11, 12 and 13) or 10 minute holding at temperatures greater than 170 F (Figures 5, 6 and 7). This phenomenon may be the result of heat denaturation of the fat globule membrane protein followed by partial desorption and migration of this copper-bearing fraction into the aqueous phase.

Data from the washed cream experiments indicated that heat-induced adsorbed copper is most tenaciously bound to the cream phase in milk heated to 180 F (Figures 10 and 11). In addition, the amount of adsorbed copper removed by washing the cream fractions was dependent upon the temperature of the wash water; more copper was lost in wash water at 80 F than in water at 36 F. The fact that a greater portion of the adsorbed copper was removed by washing cream fractions of milk flash heated to temperatures greater than 180 F, agrees with the postulation that the tenacity with which the copper-bearing membrane protein adsorbed to the fat globule interface was altered by heat treatment.

The extent of heat-induced copper migration to the cream appeared to be related to the degree of copper contamination (Figure 8) and the fat content of the milk. The level of total copper in the cream fraction was increased by storage at 34 F following heat treatment and prior to separation

(Figures 5 and 6). Slight migration of copper to the cream did take place when heated skimmilk was mixed with unheated cream. Moreover, the total copper content of the cream fraction significantly increased when copper was added to heated whole milk (Figure 7). The latter observations indicate that the greatest extent of copper migration occurred during heating, but time is an element in the attainment of copper equilibrium in the milk.

Data obtained from heat treatment of model milk systems containing milk dialyzate, washed cream and centrifugally separated micellar casein or whey protein supernatant from micellar casein, indicate that the latter two milk protein fractions bound approximately the same amount of added copper (Figure 14). Flash heat treatment of the milk system containing micellar casein resulted in the characteristic copper adsorption maximum in the cream at 180 F (Figure 14 and 17); however, the copper content of the cream fraction separated from the milk system containing the whey protein fraction increased with temperature of flash heating (Figure 14 and 18). The irreversible adsorption of copper by the cream fraction of the latter system may be due to preferential adsorption of surface-active, heat-denatured, copper-bearing whey proteins, thus masking the characteristic copper adsorption peak in the cream at 180 F. The fact that whey proteins are more easily denatured by heat than micellar casein, and that maximum

copper adsorption was not evident in the cream phase of the system comprised of whey proteins, suggests that heat denatured proteins are more strongly adsorbed at the fat globule interface than are more heat resistant proteins.

Conversely, when purified beta-lactoglobulin (BLG) was present as the only protein source in the aqueous fraction, maximum migration of copper to the cream was detected in the system flash heated to 175 F (Figure 16). Comparison of graphical data from this experiment with that for a system possessing all whey protein fractions (Figure 14) suggests that some copper bearing whey protein other than BLG exhibits more pronounced migration to the fat globule interface with increasing heat treatment. BLG is a heat sensitive protein and readily heat denatured. Possible heat-induced migration of this denatured protein fraction to the cream phase with subsequent competition for copper-binding sites may explain not only the less pronounced adsorption peak in the cream at 175 F, but also the marked increase in adsorbed copper in the cream of the control system which contained no skimmilk protein. Presumably, the added copper of the control system remained in the ionic state.

Preparation of sodium caseinate results in a more dispersed micellar structure. When this purified casein was present as the only protein source in the aqueous fraction of a model milk system, the level of copper in the cream

fraction once again increased with temperature of flash heat treatment (Figures 15 and 19). The postulation that protein denaturation is a prerequisite to heat-induced copper migration to the cream phase may not be applicable to these data. The sodium caseinate used in this milk system was undoubtedly more stable to heat than either the whey proteins or the micellar calcium casein-phosphate complex of normal cow's milk. The loss of the copper adsorption peak in the cream phase at 180 F may be explained by an increased in the pH of the milk system (above 7.0) resulting in a more polydisperse, surface-active caseinate system which migrates to and is strongly adsorbed at the fat globule interface during heat treatment of the solution.

The different heat-induced copper migration patterns for supernatant whey protein, micellar casein, sodium caseinate and BLG indicate that their individual copper-protein complexes may well have differenent reactivities and stability and the overall pattern exhibited in a given milk system would therefore be a function of not only temperature, time of heating and fat globule interfacial area, but also protein composition and quantity. Furthermore, the relative amount of copper adsorbed at the fat globule interface would be a function of the avidity of a particular protein for added copper and the molceular size of the copper-proteinate so formed.

The fact that the heat-induced copper adsorption-desorption pattern was observed in the cream fraction of a model milk system lacking skimmilk proteins (Figure 16 and 20) indicates that this phenomenon is characteristic of the fat fraction alone. The washed cream separated from this model system contained as much as 80 per cent of the total copper content of the system following flash heating at approximately 180 F, or three to four times the copper content of the cream fraction of whole fluid milk subjected to the same experimental conditions. This observation suggests that the fat globule membrane has a greater affinity for copper in the absence of milk protein and that copper-protein complexes, whatever they may be, compete for the copper binding sites at the fat globule interface as manifested through heat treatment.

Preheating fluid milk to temperatures well above those employed in pasteurization of milk has been common practice for many years in the dry milk industry. Holm et al. (33a) in 1926 showed that such heat treatment of fluid milk prior to drying improved the keeping quality of the dry whole milk. This observation was substantiated by many other research workers in this field.

Somewhat later, both Gould and Sommer (24) and Josephson and Doan (37) observed that heat treatments high enough to induce pronounced cooked flavors and liberate volatile sulfur compounds enhanced the resistance of such fluid milk to oxidation.

Oxidized flavor in cold milk does not appear until cooked flavor and sulfur odor have disappeared (or been masked). Concurrent with the production of these volatile "cooked" odors in milk is the "activation" or "liberation" of free sulfhydryl groups in milk which are capable of reacting with non-specific reagents such as sodium nitroprusside and also with the more specific reagent thiamine disulfide. From the observation that activated sulfhydryl groups in heated milk slowly disappear on standing, as indicated by a diminishing nitroprusside test and a decrease in titer of thiamine disulfide reducing substances (TDRS) and orthoiodosobenzoic acid reducing substances (IBRS), Stine (70) theorized that the heat liberated sulfhydryl groups presumably function as antioxidant in the liquid system through oxidation to some other end product.

On the other hand, Harland et al. (28a) observed that TDRS and IBRS are unchanged following storage of dry whole milk in air at 37 C. These data suggested that the reducing substances per se are not functioning as antioxidants. It might well be that the production of such activated groups merely accompanies some more significant change in the milk as it is heated. The relative ease of determining the sulfhydryl group content may result in unwarranted importance being attached to this group as a potential antioxidant in dry whole milk.

Thomas (75a) attempted to elucidate the mechanism of sulfhydryl action in milk through the addition of the sulfhydryl group blocking reagent p-chloromercuribenzoic acid (pCMB) to fluid milk stored at 40 C. He found that the addition of pCMB to fluid milk accelerated the rate of lipid oxidation. The observation that the IBRS titer remained high in milk treated with this chemical lead to the investigator's suggestion that activated sulfhydryl groups function as antioxidants in fluid milk systems.

Contrary to these findings, Stine (70) reported that the addition of pCMB, iodoacetamide (IODO) or N-ethylmalemide (NEM) often retarded the oxidation of lipid material in the dry whole milk to a variable, and occasionally dramatic extent. In addition, this investigator observed that the effect of the blocking agent in retarding the oxidative process was more pronounced in powders prepared from milks of low and intermediate preheating treatments than in those dry milks manufactured from fluid milk which had been held at 190 F for ten minutes.

Examination of the graphical representation of data obtained from experiments with whole fluid milk indicates that the greatest change in extent of heat-induced copper migration to the cream occurs during heat treatments from 165 to 175 F. Many investigators have reported sulfhydryl group activation in the non-casein protein fraction as a result of heat treat-

ment in this temperature range (19, 24, 25, 37). In order to investigate the possibility that these activated sulfhydryl groups may be involved in the copper adsorption-desorption phenomenon experienced in the cream phase, milk was treated with sufficient NEM to react with sulfhydryl groups liberated during the course of heat treatment. The observation that the presence of NEM partially inhibited heat-induced copper migration to the cream suggests that the heat-activated sulfhydryl groups were probably involved in the adsorption-desorption phenomenon (Figures 12 and 13).

The addition of NEM prior to heat treatment of model milk systems containing micellar casein (Figure 17) or supernatant from spun micellar casein (Figure 18) markedly reduced the extent of copper adsorption by the cream as a result of flash heating to temperatures from 160 to 200 F. This observation agrees with the theory postulated for whole milk systems, that heat activated sulfhydryl groups or heat induced volatile sulfides do play some role in heat-induced copper adsorption by the cream phase.

An interesting observation was made in a model system containing 2.5 per cent sodium caseinate and iodoacetamide as the sulfhydryl groups blocking reagent. In this instance, the addition of iodoacetamide to the system prior to heating not only reduced the extent of migration of copper to the cream, but also resulted in the copper adsorption-desorption

peak in the cream which failed to appear in the control model system containing no sulfhydryl group blocking agent. Possibly the presence of this chemical inhibited the previously postulated heat activated migration of some copper-bearing fraction of the caseinate from the aqueous phase to the fat interface. (Figure 19).

The addition of NEM to a model milk system free of skimmilk proteins reduced the extent of copper migration to the cream phase at all flash temperatures (Figure 20). Moreover, it may be noted that the presence of NEM resulted in the greatest inhibition of copper migration to the cream when the milk system was flash heated at temperatures below 185 F. Graphical data from this experiment suggest that NEM reacts at the level of the fat globule membrane through blockage of heat activated sulfhydryl groups at this location. In a milk system devoid of skimmilk proteins, no protein is available in the aqueous phase to complex with copper and possibly the majority of the added copper retains its ionic state, although no data are available to substantiate this. Assuming that heat liberated sulfhydryl groups in the membrane protein are chelation or adsorption sites for copper in this form, NEM may react with these loci rendering them unavailable for copper binding. The reaction of NEM with sulfhydryl groups in the membrane protein appears to be more pronounced in systems flash heated below 180 F (Figure 20). Above this temperature

possible heat induced desorption of denatured copper-bearing membrane material may be reflected in reduced inhibition of copper adsorption at the fat globule interface.

The reaction of NEM with heat liberated sulfhydryl groups in the fat globule membrane may be manifested in still another way in systems containing skimmilk proteins. Available sulfhydryl groups may be necessary as binding sites for copper proteinates that migrate to the fat globule interface as a result of heat treatment. This would attempt to explain the reduced level of copper adsorption by the cream during flash heat treatment of model systems containing skimmilk protein fractions in the presence of NEM (Figures 17 and 18). The sulfhydryl group blocking reagent iodoacetate appears to be effective in inhibiting adsorption of some unidentified copper - sodium caseinate complex by the cream when the model system was subjected to flash heating at temperatures greater than 180 F (Figure 19).

One cannot disregard the possiblity that NEM causes partial inhibition of heat-induced copper migration to the cream of whole fluid milk by reacting with heat activated sulfhydryl groups in the fat globule membrane, thus making these sites unavailable for binding of complexed, surface-active copper proteinates as well as possible ionic copper freed from its protein complex as a result of heat treatment (Figure 13).

The author suggeststhat heat liberated sulfhydryl groups in fluid milk may serve in part as antioxidants, but more significantly as binding sites at the fat globule interface for copper in one form or another. Time appears to be a factor in the extent of reaction between heat activated sulfhydryl groups and copper or copper proteinates as suggested by continued migration of copper to the cream on storage of the heated milk accompanied by gradual reduction of available -SH groups (70). Possibly a copper proteinate adsorbs at the fat interface in a manner that not only provides a physical barrier against metal catalyzed lipid autoxidation but also chelates or associates with copper in a manner that destroys or retards its oxidative catalytic activity.

SUMMARY AND CONCLUSIONS

Data from experiments employing fresh milk from individual cows indicated that added copper is preferentially adsorbed by skimmilk proteins. The cream fraction of raw whole milk adsorbed as little as 2-3 per cent of added copper following storage at 34 F for 18 hours. When this milk was heated to temperatures greater than 140 F, the copper content of the cream phase increased by as much as 600 per cent. The extent of migration of residual and added copper from the skimmilk to the cream as a result of heat treatment of the milk appears to be a function of the fat content of the milk, the total copper content and the temperature history of the milk.

Milk having a total copper content in the normal range of 40 to 240 μg Cu/l exhibited maximum and constant migration of copper to the cream phase following flash or 10 minute holding at 180-200 F. When the total copper content of the milk was increased to that level known to induce lipid autoxidation, i.e., 500-700 μg Cu/l, a copper adsorption peak was observed in the cream phase following a flash heat treatment to approximately 180 F or following 10 minute holding at 170 F. The time-temperature treatment inducing maximum adsorption of copper by the cream appears to be related to rate of reaction and, or properties characteristic of milk from an individual cow.

Heating fluid milk having a copper content greater than

500 µg Cu/l to temperatures greater than 170 F for 10 minutes or momentary heating at 180 F, resulted in desorption of copper from the cream phase. Data from washed cream experiments indicate that copper is most tenaciously bound to the cream fraction in milk subjected to that heat treatment inducing maximum adsorption of copper by the cream. The author suggests that these observations may be explained by desorption of heat altered fat globule membrane material at elevated temperatures. The copper adsorption-desorption phenomenon exhibited by the cream may also occur in milk having a total copper content of less than 220 µg Cu/l; however, the sensitivity of the analytical procedure employed may be limited in detecting this characteristic.

The fact that the amount of copper adsorbed by the cream fraction was increased by storage at 34 F following heat treatment, and that some migration of copper took place when copper was added to heated milk, suggests the following conclusions. Firstly, the greatest extent of migration of copper to the cream takes place during the course of heat treatment, and secondly, time is a factor in the attainment of copper equilibrium in the milk. Possibly, the polymorphic crystallization of milk fat is closely related to the latter factor.

Data observed from experiments with model milk systems indicate that added copper is bound primarily to the skimmilk proteins rather than the fat globule membrane. The heat-induced copper adsorption-desorption phenomenon experienced

in the cream appears to be characteristic of the cream fraction alone. Heat activated sulfhydryl groups seem to play some role in copper adsorption by the cream phase. The fact that the presence of a sulfhydryl group blocking agent partially inhibited copper migration to the cream in a system devoid of skimmilk proteins, suggests that copper is complexed in some fashion by the heat activated sulfhydryl groups in the fat globule membrane as a result of heat treatment.

The data presented do not offer a direct explanation as to why the heat treatment of milk to temperatures greater than 165-170 F confers antioxygenic properties to the milk. Indirect evidence does indicate that the copper-protein complexes, whatever they may be, are altered in some manner as indicated by the adsorption-desorption phenomenon described in this thesis. Possibly some copper-protein complex(es) adsorbs at the fat globule interface in a manner that presents not only a physical barrier against metal catalyzed autoxidation, but also sequesters or associates with copper in a mode that inhibits its ability to breakdown hydroperoxides to free radicals.

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APPENDIX

I. Preparation of Glassware.

To avoid extraneous copper contamination, all glassware used in sampling, heating, separation, freeze-drying, and copper determinations was prepared for use by complete immersion in concentrated nitric acid followed by a five fold rinsing in deionized, redistilled water having an electrical conductivity of less than 0.9 μ mhos.

II. Standard Copper Solution. 1 mg Cu/ml 0.5000 g electrolytic sheet copper is dissolved in 20 ml 6 N nitric acid and evaporated almost to dryness. Several drops of glacial acetic are added, and the solution is quantitatively transferred to a 500 ml volumetric flask and made to volume with redistilled water.

(1) Working standard copper solution. 1 μg Cu/ml. 10 ml of the standard copper solution is diluted to 100 ml; after mixing thoroughly, 10 ml of this intermediate solution is diluted to exactly 1000 ml, yielding a solution containing 1 μg Cu/ml. This solution should be prepared each time a standard curve is made or for controlled copper addition to milk systems, and should be used immediately after preparation. Otherwise, losses may occur due to adsorption of copper the surfaces of glassware.

III. Copper Determinations.

(1) Preparation of Standard Curve.

100 ml aliquots of thoroughly mixed fresh raw milk are treated with the working copper solution in amounts ranging from 2 to 12 µg Cu per 100 ml milk. These prepared standard milks are frozen, freeze-dried, ashed in platinum crucibles at 550 C for 10 hours, and the copper content of the resulting ash determined by the method that follows, using the residual copper in 100 ml of the original raw milk as an appropriate blank in the copper determinations.

(ii) Reagents:

a. Ammonium Citrate. 430 g dibasic ammonium citrate are dissolved in 300 ml redistilled water. 200 ml concentrated ammonium hydroxide are added to the citrate solution and the total volume made up to 1000 g. Copper contamination is removed by adding 10 mg sodium diethyl dithiocarbamate and extracting the solution with three 100 ml portions of analytical grade carbon tetrachloride.

b. Redistilled ammonium hydroxide.

c. 2 per cent aqueous solution of sodium diethyldithiocarbamate. This solution is prepared fresh and used immediately after preparation.

d. Carbon tetrachloride, A. R. grade.

e. 1 per cent ethanolic phenophthalein.

f. 2·4 N Hydrochloric acid.

(iii) Procedure.

a. The ashed freeze-dried milk or milk fractions

are removed quantitatively from the platinum crucibles with 10 ml boiling 2.4 N HCl and transferred to 100 ml extraction flasks fitted with glass stoppers. The remaining ashed material is rinsed from the platinum crucibles with two additional 5 ml portions of boiling 2.4 N HCl.

b. 15 ml of the ammonium citrate buffer is pipeted into the extraction flask, followed by one drop of 1% ethanolic phenolphthalein solution. Redistilled ammonium hydroxide is added to the flask contents until a faint pink endpoint is obtained (pH about 9).

c. 2 ml of 2% aqueous sodium diethyldithiocarbamate solution is added to the flask. The flask is shaken vigorously for 2 minutes and allowed to stand for 15 minutes until the reaction has gone to completion.

d. Exactly 10.0 ml of carbon tetrachloride is pipeted into the flask, the flask is shaken vigorously for 5 minutes and allowed to stand for 10 minutes for complete solvent separation.

e. Approximately 4 ml of the lower yellow solvent layer is removed by plunging a 4 ml pipet through the upper aqueous layer. The optical density of the removed solvent is determined in a Beckman DU spectrophotometer at a wave length of 435 mμ with a slit width of 0.06 mm.

f. A blank is prepared for each group of samples using 20 ml of 2.4 N HCl instead of the ash solution.

IV. Preparation of Purified Casein. (8a)

(i) Five one-gallon portions of fresh skimmilk are treated with 0.5 N HCl until the pH of the milk is adjusted to and maintained at pH 4.6-4.8. The resulting precipitate is dissolved in 0.5 N NaOH and reprecipitated with 0.5 N HCl at a pH of 4.8. The supernatant liquid is decanted and the residue from the five individual batches combined into one volume.

(ii) The residue is mechanically dispersed for 5 minutes in redistilled water and the dispersion filtered through several layers of cheese cloth. This washing procedure is repeated a total of four times.

(iii) The fourth casein dispersion is filtered through Whatman No. 1 filter paper in a Buchner funnel at a suction pump. The collected residue is washed twice with 95% ethanol for 5 minutes. After each washing the ethanol is removed by vacuum filtration.

(iv) The casein residue is redispersed in absolute ethanol and filtered under suction a total of three times.

(v) The casein residue is washed under continuous agitation for a period of five minutes in petroleum ether and again filtered under suction. This procedure is repeated.

(vi) The casein residue is finally washed four consecutive times in redistilled water followed by suction filtration. Following the fourth washing, the casein is put into colloidal dispersion with 0.5 N NaOH; the resulting sodium caseinate

sol has a final pH of 7.8.

(vii) The sodium caseinate sol is transferred to a semi-permeable cellophane membrane and dialyzed for 24 hours against 10 gal of distilled water maintained at 34-36 F. The dialysis is repeated against two additional 10 gal volumes of distilled water under the conditions described.

(viii) The dialyzed material is carefully removed from the membranes, transferred to shallow procelain trays, frozen at -10 F and freeze-dried.

(ix) The freeze-dried sodium caseinate is stored in air-tight bottles under refrigeration.

V. Preparation of Iodoacetamide. (1a)

(i) Materials:

alpha-chloroacetamide (Eastman Kodak)

Sodium iodide

Acetone

(ii) Procedure:

50 g alpha-chloroacetamide and 80 g sodium iodide are dissolved in 1 l. acetone. The solution is allowed to stand in the dark at room temperature for five days. The precipitate (Sodium chloride) is removed by filtration and the acetone is removed by distillation under reduced pressure until the vapor temperature begins to rise rapidly. At this point, the flask and contents are quickly cooled in an ice water bath. The crystals of iodoacetamide are filtered off

in the cold, and are washed with ice cold acetone.

The iodoacetamide is purified by dissolving it in acetone, removing most of the solvent by distillation and recrystallization in the cold.

The iodoacetamide is recrystallized a total of three times from acetone. The crysatls are dried in a current of air, and are dissolved in an equal weight of water by warming rapidly. The aqueous solution is cooled rapidly in ice water and filtered in the cold. The crystals are dried in a desiccator and the recrystallization from aqueous solution is repeated.

Melting point of the crystals is approximately 95.5 C.

Iodoacetamide is slowly converted to iodoacetic acid in neutral and alkaline solution and slowly liberates iodide in acid solution. The pure crystals are stored in an amber bottle to retard decomposition; if the product reddens with age, it is recrystallized before use.

VI. Nitroprusside Test.

The procedure described by Josephson and Doan (37) was employed.

(i) Reagents:

Solid ammonium sulfate, A.R.

Sodium nitroprusside solution, 4.5%

Solution in distilled water, prepared with reagent grade

sodium nitroprusside.

Concentrated (28%) ammonium hydroxide, A. R.

(ii) Procedure:

A 5 ml sample of milk is saturated by adding excess solid ammonium sulfate in a test tube and shaking. Five drops of a 4.5% solution of sodium nitroprusside (freshly made) are introduced with agitation followed by 5 drops of concentrated ammonium hydroxide. After again shaking the tube contents, the relative color intensity is noted.