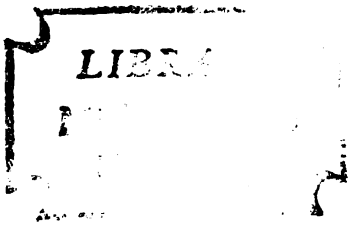
A STUDY OF LIPID OXIDATION IN MODEL SYSTEMS OF COPPER-PROTEINS, MILK LIPIDS AND MILK DIALYZATE

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

JASWANT SINGH AULAKH

1967



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thesis entitled

A Study of Lipid Oxidation in Model Systems of Copper-Proteins, Milk Lipids and Milk Dialyzate

presented by

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has been accepted towards fulfillment of the requirements for

Ph.D. degree in Food Science

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Date January 5, 1968

ABSTRACT

A STUDY OF LIPID OXIDATION IN MODEL SYSTEMS OF COPPER-PROTEINS, MILK LIPIDS AND MILK DIALYZATE

by Jaswant Singh Aulakh

The binding of copper with micellar casein, fat-globule membrane protein, α-casein, β-casein, β-lacto-globulin was studied by the technique of equilibrium dialysis at pH 6.5. Solutions of these proteins, in concentrations of 2.000, 3.000, 2.436, 1.205, and 1.420 mg/l were placed in washed dialysis bags and dialyzed against each of four copper solutions for 72 hours, an interval considered sufficient for the attainment of equilibrium. The concentrations of the four copper solutions outside the dialysis bags were 16x, 32x, 64x, or 80 x 10^{-6}M, respectively. The data were represented in graphs by plotting the moles of bound copper per mole of protein against the logarithm of the concentration of unbound copper. The moles of copper bound per mole of protein were approximately 67, 24, 10, 2, 2.5, respectively, for the above proteins at a free copper concentration of 1 x 10^{-5}M. The results clearly show the great difference in ability of certain milk proteins to bind copper ions. Micellar casein bound the greatest amount of copper.

The fat-globule membrane protein demonstrated the second greatest affinity for copper ions. Much lower amounts of copper were bound by α-casein, β-lactoglobulin, and β-casein.

Model systems of copper-micellar caseinate, copper-fat-globule membrane protein, copper-sodium α-caseinate, copper-sodium β-caseinate and copper-β-lactoglobulin were incorporated into model systems of washed fat-globules and milk dialyzate. The model system without copper was specified as the control. The systems with copper and without copper were heated at 145°F for 30 minutes and 190°F for 10 minutes. Model systems of copper proteinate of β-lactoglobulin, fat-globule membrane protein and a model system of washed fat-globules and milk dialyzate were also heated individually at 145°F for 30 minutes and 190°F for 10 minutes and then combined together. Samples were stored at 35°F for 10 days. At 2-, 4-, 6-, 8-, and 10-day intervals, oxidation was followed by determination of thiobarbituric acid and peroxide values. The results indicated that heated systems showed significantly less tendency to oxidize than unheated systems. Systems heated to 190°F for 10 minutes exhibited the least amount of oxidation. The individual heating of copper proteinates and model system of washed fat-globules and milk dialyzate demonstrated lower level of oxidation than the systems in which copper proteinates

were heated and washed fat-globules and milk dialyzate were unheated. The decrease in oxidation in such systems suggests that physical or physico-chemical changes at the fat-globule surface are associated with the beneficial effects of high heat treatment of milk.

A STUDY OF LIPID OXIDATION IN MODEL SYSTEMS OF COPPER-PROTEINS, MILK LIPIDS AND MILK DIALYZATE

By

Jaswant Singh Aulakh

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science

1967

DEDICATION

The work in this thesis is dedicated to my beloved wife, Gurcharan Kaur Aulakh, whose patience and encouragement during this strenuous period of graduate study will never be forgotten.

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. C. M. Stine, Associate Professor of Food Science, for his encouragement and counsel during this study. His philosophy of life as well as his scientific competence will be a perpetual source of inspiration. The author also extends his thanks to Dr. H. A. Lillevik, Dr. J. R. Brunner, Dr. E. J. Benne and Dr. T. I. Hedrick for their suggestions in the preparation of this manuscript.

The author further thanks and is grateful to Experiment Station of Michigan State University for financial assistance in the form of an assistantship.

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INTRODUCTION

Milk lipids are generally recognized as the major contributors in the development of oxidized flavors in dairy products. The reaction of these lipids with oxygen, resulting in flavor deterioration, creates serious problems in storage stability. The impairment of quality, due to autoxidation, is widespread, and manifests itself in many ways, depending upon the product involved. In certain products, particularly dry whole milk, autoxidation has been a major impediment to the development of an acceptable product. Considerable expense and effort have been expended in attempts to prevent autoxidation by use of antioxidants and inert gas packing, but such methods are only partially effective.

Copper is capable, to a great extent, of catalyzing these oxidation reactions. Much information on the distribution of copper in milk is now available in the literature. Copper complexes with the proteins in the fluid milk systems and the copper-protein complex in turn is capable of catalyzing the development of oxidized flavor.

In this investigation, binding of copper with various milk proteins was studied. The objective of this

study was to determine the effect of low and high heat treatments on the various copper-proteinates suspended in a model system of washed fat-globules and milk dialyzate.

LITERATURE REVIEW

Oxidized flavor in some dairy products, namely fluid milk, ice cream, and dry whole milk is a distressing problem to the dairy industry. No endeavor has been made in this thesis to extensively review the vast literature on oxidized flavor unless it pertained specifically to distribution of copper in fluid milk, the binding of copper with milk protein, and the role of copper in the development of oxidized flavor. Comprehensive general reviews of the subject of oxidized flavor include reviews by Brown and Thurston (9), Day (20), Greenbank (36), Pont (85), Riel and Sommer (88), Stull (106), and Wilkinson (120).

Distribution of Copper in Fluid Milk

Elvehjem et al., (27) reported earlier that cow's milk contained about 0.15 mg of copper per liter. The copper content of cow's milk could not be increased by feeding the cows sufficient copper sulfate to increase the copper intake five fold. The average copper content of fresh cow's milk was found to be 0.0553 ppm $\pm$ 0.0046 (79). No considerable difference was noted between the copper contents of samples from grazing cows and those from stall fed cows. The average copper content of raw

bottled whole milk was 0.0820 ppm ± 0.0084, and of heat treated bottled whole milk, 0.0995 ppm ± 0.0063. Somewhat higher copper values were found in skimmilk, buttermilk and cream, butter and cheese.

Koppeljan and Mulder (63) determined the copper content of the milk of a number of cows at different times in a lactation period. Generally a considerably higher amount of copper was found at the beginning of a lactation period than in the second half. The colostrum milk from most cows contained less copper than milk obtained during early lactation. Similar results were reported by King and Dunkley (52). They contended that a relatively high concentration of natural copper was present in early lactation milk, and a level of about 0.02-.04 ppm for most cows during the remainder of the lactation period. No difference was observed in the effect of pasture and dry feed on the natural copper concentration of milk from cows on these rations. According to Mulder et al. (76), the natural copper content of normal cow's milk was 20-40 ug/kg, with somewhat lower or higher values occurring at the beginning of lactation. The influence of a number of factors, e.g. variation during one milking, influence of the season and individual effects on copper content of the milk were also discussed (76). The colostrum contained a rather high amount of copper (100 ug/kg). In the first day after calving, the copper content of the

milk increased, a maximum being reached after 0-7 days (about 200 ug/kg), and thereafter the copper content decreased to normal values (20-40 ug/kg). Mulder et al. (76) did not provide a satisfactory explanation of such changes and furnished little information on the influence of the copper content of the cow's feed and of intravenous injections with copper solutions, although many experiments on this topic have been reported in the literature (52). The authors concluded that the copper content of the cow's feed had no influence on the copper content of the milk.

Rice and Miscall (87) showed that on passing milk containing copper through a separator, the metal distributed itself between the cream and skimmilk fractions approximately in proportion to the water content of these fractions. The copper was considered, therefore, to be dissolved in the water. Their results indicated that the copper did not go with the fat and was therefore not dissolved in the fat. Davies (19) reported that with gravity creaming the copper was distributed in direct proportion to the water in the two fractions or in direct proportion to protein nitrogen. The copper was not distributed according to the weight of the fat. In the centrifuged portion where the residual fat in the separated milk layer was too small to be determined accurately, the copper/protein nitrogen ratio was approximately three

times greater in the cream layer. The high copper-protein nitrogen ratio in the cream layer was apparently due to the adsorption of copper at the surface of the fat-globules.

Dills and Nelson (22) and Allen (2) revealed that iron and copper did not exist in the ionic state in milk and related products, but were combined in some way with the lipo-protein complex which surrounds or is at least adsorbed on, the fat-globules. This suggests that the catalytic action of these metals must be considered as the effect of the combined metal proteinate, and not as the effect of a metallic ion. Davies (19) observed that the concentration of the ionic form of heavy metal in milk was of a low order but increase in acidity caused more ionic metal to form. Willard and Gilbert (116) recovered more copper from the cream than from the skimmilk fraction and oxidized flavor development was correspondingly more pronounced in the cream fraction. However, Miller and Tracy (74) showed that copper in milk products was associated with the non-fat portion rather than the fat phase. Butter made by the continuous process contained less copper than the original cream. The centrifugal treatment of cream in the process of making butter by the continuous method resulted in a concentration of copper in the non-fat portion. Similarly, butter oils made by the centrifugal process in the continuous method of making butter contained only minute amounts of copper.

Mulder and Koppeljan (77) indicated the uneven distribution of the naturally occurring copper between the different phases of milk. A high proportion was retained on the surface of the fat-globules, the proportion varying with the cow and the stage of lactation. Naturally occurring copper was found to be associated with fat-globule membrane, whereas the remaining copper in the separated milk was closely combined with the proteins (72, 73). King et al. (54) reported that natural copper was concentrated at the surface of fat-globules but most of the added copper was uniformly combined with skimmilk proteins. Only 2-3% of the total added copper was associated with the fat-globules. Natural copper could not be dialyzed, although added copper in the skimmilk could be slightly dialyzed. However, added copper associated with fat-globules was non-dialyzable. King and William (53) in their later studies used Cu^{64} in determining the distribution of natural copper in milk at intervals during early lactation. The amount of copper bound with fat-globules was about 15% after 2 and 4 weeks of lactation and 35% after 10 weeks. The change in copper distribution brought about a change in susceptibility to oxidized flavor. Washing of cream and chelation by ethylenediamine tetraacetic acid (EDTA) removed up to 90% of lipid-bound copper. When these treated fat-globules were re-suspended and recovered from the original skimmilk, their copper content increased.

Samuelsson (93) very recently investigated the distribution of added and natural copper by adding activated copper chloride to milk. The milk was then fractionated and the copper content in these fractions determined by means of activation analysis. Results showed that about 90% of the added copper was combined with skimmilk proteins and about 10% with fat-globules. About 5% of the copper could be washed from the fat-globules and about 5% was bound with the actual membrane proteins. Within the skimmilk proteins the whey proteins contained the greatest amount of copper per gram protein. Similarly, Hartman (42) reported that whey proteins had the greatest affinity for copper; 15 to 20% of the natural copper was associated with the fat-globules, 10-15% could be washed off and the actual membrane protein contained about 4% of the total copper but at the same time contained the highest copper content per gram protein (93).

Binding of Copper with Milk Proteins and the Methods Used in the Binding Studies

Vandevelde (115) studied the precipitation of milk proteins by copper salts. The results indicated that only the cation was bound by the milk proteins, and this in varying quantities, depending upon the concentration of copper solution employed. Sollner (102) used collodion membranes of high ionic selectivity for the potentiometric determination of K+, Na+, Li+, NH_4+, Mg+ and

probably other cations. The potential difference which arose between a known solution on the one side of the membrane and the solution of unknown concentration on the other side of the membrane was utilized to determine a particular ion. This method could be used in studies of the activity of counter-ions in colloidal systems, for the determinations of the anion and cation binding capacity of proteins, and similar problems. Leviton et al. (71) reported the binding of riboflavin and riboflavin phosphate by the proteins of milk, particularly α- and β-casein. They determined binding constants and calculated thermodynamic quantities. The binding was of low order of magnitude with an extremely high temperature coefficient. The binding by the calcium caseinate-phosphate complex in milk was equal to the binding by the component protein molecules. They found certain similarities between the binding by the caseins and the binding by flavo-protein enzymes. Other workers (105) studied the stoichiometry of the biuret reaction of alkaline copper tartrate with proteins, including serum proteins, β-lactoglobulin, edestin, pork gelatin, gliadin and zein. In all of these proteins except serum globulin, six peptide nitrogens were bound by chelation to each copper. In serum globulin, five peptide nitrogens were combined with each copper. The authors also discussed the contributions of cysteine, tyrosine, glutamic acid, aspartic

acid, histidine, arginine, and lysine to the color of copper-protein complexes. They proposed methods for determining peptide nitrogen by means of copper binding and for estimating proteins by a titrimetric method.

Klotz and Curme (60) and Klotz et al. (61) studied the extent of binding of cupric ions and methyl orange by bovine serum albumin at a pH of 5.67 and 4.8, respectively. The equilibrium dialysis technique was used in the binding studies. The decrease in binding with decrease in pH and the character of the absorption spectrum of the copper-albumin complex indicated that carboxyl groups on the protein were involved in the bond with the cation. Later on, Klotz (59) reported the binding of cupric ions and calcium ions by bovine serum albumin. Pronounced differences existed in the behavior of Cu++ and Ca++ with albumin near its isoelectric point. Copper was bound strongly and calcium hardly at all. Copper formed much stronger complexes with either the carboxyl group of acetate or the nitrogen of ammonia than did calcium or magnesium. The metal-binding activity of conalbumin has also been reported by Fraenkel-Conrat and Feeney (15). Equilibrium dialysis experiments showed that the copper was less firmly bound than iron.

Carr (10, 11, 12, 13) and Carr and Woods (14) used membrane electrodes while studying the binding of small ions in protein solutions. To make a measurement,

the protein solution containing calcium chloride was placed inside a bag-shaped sulfonated polystyrene collodion membrane, and a known amount of distilled water was placed outside. A concentrated standard solution of $CaCl_2$ was added to the outside solution. After each addition the membrane potential was measured, and this procedure was continued until the potential reversed in sign. The potential measurements and the concentration of the outside solution were plotted on the semi-logarithmic graph paper. The concentration of the calcium in the outside solution could be calculated from such a graph. The amount of calcium bound to the protein was then calculated directly from the difference in the concentration of calcium in the outside solution and the concentration of calcium added to the protein solution. Baker and Saroff (4) used an apparatus consisting of three chambers in a linear array, separated in pairs by two permselective membranes, one negatively charged and the other positively charged. The protein solution (ß-lactoglobulin) was placed in the center chamber and accurately known solutions in the end chambers. A potential was created across each membrane, linearly related to the logarithm of the activity of the free Na^+ or Cl^- in the protein solution. Equilibration of the solutions required from 5 to 10 minutes. The potentials were recorded and a linear interpolation to zero potential

on a graph of millivolts versus log Na+ or Cl^- gave the log free Na+ or Cl^-. The difference between Na+ added and free Na+ measured was the Na+ bound to the protein.

Methods of Copper Analysis in Milk and Milk Products

Most methods for the estimation of copper are based on dry-ashing procedures, followed by colorimetric measurement of copper as its carbamate (23, 47, 52, 74). Many modifications of carbamate (74, 1, 5, 16, 23, 47, 64, 46, 82, 107, 117), and dithizone (75) methods have been reported in the literature. Duin and Brons (25) described a simple and reliable method for the estimation of the copper content of milk and milk powder. This method was a modification of the rapid method (24) of determining copper by using carbon tetrachloride and trichloroacetic acid. The new method was quite accurate and could be used as a routine method for the analysis of milk and milk powder. Several workers (37, 80) recently demonstrated the improved acid precipitating methods. King and Black (55, 56) made a comparison of the determinations of copper in butter and butterfat by a wet ashing colorimetric method, by atomic-absorption spectroscopy and by neutron activation analysis. The wet ashing colorimetric method gave the most consistent results and these were in agreement with those from atomic absorption. Gehrke et al. (33) developed a rapid, reliable and

accurate spectrographic method for the quantitative determination of tin, copper, iron, and lead in milk and evaporated milk. The analyses of all four metals could be made simultaneously on a single sample. The precision of the method for the respective elements was from ± 5 to ± 8%.

Willis (119) determined copper in butter and butter oil by atomic absorption spectroscopy. The method was accurate in determining low levels of copper and according to the author this method could be suitable for determining small amounts of copper in milk. Samuelsson (94) determined trace amounts of copper in milk by neutron activation analysis. Smith (97) very recently developed two rapid methods of precipitation for determining the copper content of the milk and compared these methods with a dry ashing procedure. The methods involved the use of zinc dibenzyl-dithiocarbamate (Arazate) which requires no pH adjustment. In comparison to dry-ashing procedures, considerably less time was involved for the analyses and both methods were precise. Simultaneous micro-determination of copper and iron using mixed phenanthrolines has also been reported in the literature (121, 122).

Lipid Oxidation in Fluid Milk Induced by Copper

Early investigations (18, 21) reported on the development of an oily metallic off-flavor in raw and

heated milk. The off-flavor was traced to the presence in the milk of copper and its compounds and was prominent in milk exposed to copper during flash pasteurization. A protective milk coating which formed on metal at temperatures above 75°C prevented direct contact of milk with metal. Several workers (26, 9) demonstrated that under some conditions, dissolved copper combined with stainless steel in a form that was readily available for contamination of milk. Absorption of dissolved copper on stainless steel occurred greatly in the pH range of 6 to 10. The amount of copper that absorbed increased with the concentration of copper in solution, the temperature, and the time of exposure. Krukovsky and Guthrie (66) demonstrated the promotion of the tallowy flavor in milk by adding copper in the presence of ascorbic acid. William and Burgwald (118) studied the development of oxidized flavor in milk when copper at a concentration of 2 ppm was added after pasteurization of the milk at 143°F for 30 minutes. Copper was an effective catalyst for the development of oxidized flavor in milk and the intensity of the flavor was greater when copper was added after rather than before pasteurization (36). Pont (85) demonstrated oxidized flavor in whole milk containing 1.0 ppm added copper after 2-3 days of storage at 2-5°C. He concluded that oxidized flavor in milk was made up of an oily-metallic component,

produced by oxidation of fat, and a cardboard component associated with skimmilk. Mulder et al. (78) reported that oxidized flavor was not detected in any milk containing no added copper after 2-3 days storage, while a slight off-flavor developed in only a few samples after 6 days storage. The susceptibility to copper-induced oxidized flavor varied markedly from cow to cow.

Ascorbic acid plays a crucial role in the development of copper catalyzed oxidized flavor in milk. An extensive amount of work concerning this topic has been reviewed in literature by several investigators (31, 32, 39, 40, 42, 43, 68, 83, 84, 99, 104, 113, 114, 57). Corbett and Tracy (17) reported that although ascorbic acid reduced oxidized flavor development in some milks to which copper had been added, it extensively increased the flavor in others. This was confirmed by Olson and Brown (81). They showed that washed cream from susceptible milk did not develop flavor when contaminated with copper ions and stored at 40°F but the addition of 40-200 mg/liter of ascorbic acid plus copper caused a strong oxidized flavor to develop. Brown and Olson (7) also found that resistant milk could develop the flavor following addition of copper and ascorbic acid or dehydroascorbic acid to the milk. Similarly, Krukovsky and Guthrie (66, 67), found that milk depleted of its natural ascorbic acid failed to develop off-flavor but

the flavor could be induced by the addition of reduced or oxidized form of ascorbic acid. Pont (85) reported that cardboard and metallic flavors could be induced in skimmilk, as well as whole milk and washed cream, by addition of ascorbic acid.

Recently King and Dunkley (52) demonstrated that the levels of copper naturally present in milk are sufficient to catalyze the development of off-flavor and that there was a strong correlation between the incidence of spontaneous development of oxidized flavor and high levels of natural copper in the milk. Smith and Dunkley (98, 101) emphasized the role of ascorbic acid in reducing copper to the cuprous state which was considered the active catalyst for phospholipid oxidation.

In more recent studies El-Negoumy (28) showed that in the absence of salts, (sodium citrate, NaCl, KCl and $CaCl_2$) ascorbic acid behaved as an antioxidant at concentrations from 0.50-2.0 mg and a weak pro-oxidant at concentrations from 2-10 mg per 100 ml. The reverse of this behavior was observed in the presence of salt solutions, with variation of oxidative intensity depending upon the composition of the model system.

Roland et al. (92) and Roland and Trebler (91) developed a method for determining the relative sensitivity of milk and cream to oxidized flavor induced by pasteurization in the presence of metallic copper. The

sensitivity of standaridized milk and cream to copper-catalyzed oxidized flavor was apparently related to the composition of the products as determined by the fat content. A variation of about one % in the whole milk caused a significant change in the flavor score. The mechanical separation of milk produced a significant decrease in its sensitivity to copper-catalyzed oxidized flavor.

Thurston et al. (111) found that homogenization, prolonged agitation at low temperature, and freezing and thawing of milk eliminated susceptibility to development of oxidized flavor. The most likely theory to explain the non-appearance of oxidized flavor in homogenized milk was the increased absorption of protective protein on the surface of the fat-globule (112). Larsen et al. (69) showed that homogenization of milk had a tendency to stabilize the milk against oxidation but did not increase or decrease the oxidation-reduction potential. The mechanism by which homogenization retarded the development of oxidized flavor was not associated with oxidation-reduction potential. The homogenized milk was relatively immune to oxidized flavor, since in such milk the fat remains scattered and the unstable lipid component of the fat-globule membrane is split and withdrawn from the surface into the interior of the fat-blobules, where it is protected by the antioxidant (65). Guthrie (38)

studied the development of oxidized flavors in unhomogenized and homogenized milk. None of the homogenized samples was judged to be slightly oxidized, but a very few were doubtful. In contrast, one-half of the unhomogenized samples developed oxidized flavor by the 5th day of storage. Tarassuk and Koops (109) reported that the site of oxidative reaction that produces oxidized flavor in milk was the surface of fat-globules and the reactants were phospholipids and oxygen. These reactions were catalyzed by the copper-protein complex. The concentration of the phospholipids and the copper-protein complex per unit of surface area was decreased proportionally to the homogenization pressure. This decrease in concentration per unit of the newly formed fat-globule surface was apparently the most important factor that retarded the development of oxidized flavor in homogenized milk.

Brown et al. (8) demonstrated that the contamination of the milk after pasteurization with amounts of copper varying up to 2.5 ppm caused a more frequent and more intense development of flavor than did contamination with identical amounts of copper before pasteurization. When amounts of copper ranging up to 2.5 ppm were added to susceptible milk, the intensity of the resulting oxidized flavor was greater in the raw milk than in the corresponding pasteurized milk where the copper contamination occurred before pasteurization. Gould and Sommer (35)

reported that the cooked flavor was closely related to heat induced inhibition of oxidized flavor if copper contamination occurred before heating, but no relation was found when copper was added after heating. Gjessing and Trout (34) exposed milk to 65°C or to 70°C for 10 minutes, and then added copper. A distinct oxidized flavor developed when copper was added before heating. At the 75, 80 and 85°C exposures little difference was noted in the intensities of the developed oxidized flavor, which required the presence of 0.26 to 0.39 mg of copper per liter to develop a distinct oxidized flavor, regardless of whether the copper was added before or after heating. At 90°C, however, the oxidized flavor was not observed in the milk contaminated with 0.52 or 0.65 mg of copper per liter before heating, but developed slightly when similar amounts of copper were added after heating.

Ewbank and Gould (29) found that cream containing 5 ppm of added copper and heated at 85°C flash and 90.6°C flash, produced butter oil of stability equal to that of a control pasteurized at 62.8°C for 30 minutes and containing no added copper. The stabilizing effect of temperatures of 85°C and 90.6°C was about equal regardless of the addition of copper before or after heating. This heat effect may be the result of the formation of hydrogen sulfide and sulfhydryl groups which inactivated

the copper sufficiently to obviate its full catalytic action. Casein-copper and lactalbumin-copper mixtures were heated at 121°C for 15 minutes by Bernhart and Linden (6). They added both heated and unheated mixtures of ascorbic acid solution and found that heated mixtures caused a lower rate of loss of reduced ascorbic acid than did the unheated ones. When copper was added to previously heated protein solution, an effect on reduced ascorbic acid oxidation intermediate between heated and unheated samples occurred. They concluded that high temperature heat treatment retarded pro-oxidant effects of milk copper.

Smith and Dunkley (100) showed that the susceptibility of raw bulk milk to oxidized flavor was increased by pasteurization at 73°C for 40 seconds and decreased by homogenization and laboratory heat treatment at 75°C for 30 minutes. Studies with chelating agents indicated that no correlation existed between catalysis of lipid oxidation and copper catalyzed oxidation of ascorbic acid. The effect of heating on the distribution of added copper in milk was studied by Sargent (96). Only 2-4% of the added amount of copper was associated with the fat-globules at 1°C while heating to 60°C enhanced this association by 600%. The extent of this migration of copper in milk was dependent upon the fat content, the total copper content and the temperature to which milk was exposed. In very

recent studies, Samuelsson (95) reported that upon heating whole milk, the copper content of the cream more than doubled as the 10 minutes heating temperature rose from 50 to 80°C. A particularly sharp increase was observed between 65° and 75°C. When whole milk was heated at 80°C for 10 minutes, the changes in copper content in various fractions were observed.

EXPERIMENTAL METHODS AND MATERIALS

Preparation of Various Milk Proteins

Sodium α- (crude) and β-caseinates were prepared according to the method of Hipp et al. (48). The whole casein was dissolved in concentrated aqueous urea solutions (6.6M), and separated into the components α- and β-casein by adding water in appropriate amounts. Sodium α- and β-caseins were obtained by dissolving α- and β-casein in .1N NaOH solution followed by freeze drying. β-Lactoglobulin was obtained from whole milk following the method of Aschaffenburg and Drewry (3). Fat-globule membrane protein (soluble fraction) was prepared from cow's milk according to the method of Herald and Brunner (45).

Micellar casein was prepared by centrifuging skim-milk.

Procedure for Binding of Copper with Proteins

Seamless visking cellulose tubing of 9- or 10-inch lengths (0.984 inches flat width) were soaked three times in redistilled water. Ten milliliters of protein solution of known concentration was transferred accurately

through a length of the tubing which had been tied at one end. The bag was suspended in a 25 ml glass cylinder containing 10 ml of copper solution. A control dialysis bag containing buffer of pH 6.5 was similarly prepared and suspended in a glass cylinder containing copper solution. The two glass cylinders were placed in cold room at approximately 35°F for a period of 72 hours, an interval sufficient for the attainment of equilibrium (60). The bags were then removed and the external solution analyzed spectrophotometrically for copper (96). The extent of binding of copper ions by the different proteins under investigation was calculated from the difference in the concentration of free copper in a control bag containing buffer and the one containing protein. The degree of binding was determined from a plot of the average number of moles of bound copper per molecule of protein versus the logarithm of the concentration of the free copper ion (60).

Preparation of Model Systems

Milk Dialyzate

Several seamless visking cellulose tubings 3.14" (flat width) were filled with 1 L of redistilled water. The dialysis tubings were sealed by tying a knot and were suspended in 10-gallon stainless steel cans containing raw whole milk. One 10-gallon can contained

only one dialysis bag. The cans were kept in a cold room at a temperature of 35°F for 36 hours, an interval sufficient for equilibration of water with the milk.

Washed Fat-Globules

Eight gallons of raw whole milk were separated at 40°C, employing a laboratory model DeLaval cream separator. The cream was washed four times with four volumes of 40°C distilled water.

Preparation of Samples and Heat Treatment

Four grams of washed fat-globules were added to 100 ml of milk dialyzate ($4\%\frac{W}{V}$) in order to obtain a model system of a composition similar to normal cow's milk. To ensure thorough mixing, the mixture of fat-globules and milk dialyzate was carefully shaken. Model systems of different copper proteinates under study were suspended in a model system of washed fat-globules and milk dialyzate. The model system without copper was designated as the control. The model systems with copper and without copper were transferred to a 1 L erlenmeyer flask fitted with an immersion thermometer and were subjected to heat treatments of 145°F for 30 minutes and 190°F for 10 minutes. During heating the model systems were mixed continuously. After cooling in ice cold water, the samples were stored at 35°F for 10 days.

Oxidation Studies

At intervals of 2, 4, 6, 8, and 10 days the samples were removed from the cold storage and equilibrated to room temperature by placing them in a warm water bath. Oxidation was followed by performing TBA and peroxide value tests. The optical density (OD) of the TBA reaction mixture was measured at 532 mμ by the method of King (58). Blank determinations were made on all the reagents and their OD values subtracted from those of the oxidized samples. The fat sample for the peroxide test was prepared as described by Pont (84) and analyzed by a modified ferrous thiocyanate test (103).

Experimentation

Experiment I.--Binding of Copper with Micellar Casein

Ten milliliters of copper solutions of the following concentrations, 80 x 10^{-6}M, 64 x 10^{-6}M, 32 x 10^{-6}M, and 16 x 10^{-6}M were accurately measured successively into four 25 ml graduated glass cylinders. A dialysis bag containing 10 ml of micellar casein solution (pH 6.5) at a concentration of 2.000 mg/l was placed in each cylinder. Control dialysis bags containing 10 ml of buffer (pH 6.5) were also kept in glass cylinders containing copper solutions of above-mentioned concentrations. Both sample and control cylinders were placed in cold room at a temperature of 35°F for 72 hours. The bags

were then removed and the external solution analyzed for copper ions. The data were represented in a graph and the moles of copper bound per mole of micellar casein were determined.

Experiment 2.--Binding of Copper with Fat-globule Membrane Protein

The concentration of the fat-globule membrane protein solution used in this experiment was 3.000 mg/l. The rest of the procedure was as described in the preceding experiment. The data were presented in a graph and the moles of copper bound per mole of fat-globule membrane protein were read from the graph.

Experiment 3.--Binding of Copper with Sodium α-Caseinate

The concentration of the sodium α-caseinate solution used in this experiment was 2.436 mg/l. The rest of the procedure was similar to that of Experiment I. The data were represented in a graph and the moles of copper bound per mole sodium α-caseinate were determined.

Experiment 4.--Binding of Copper with Sodium β-Caseinate

The concentration of the sodium β-caseinate solution employed in this experiment was 1.205 mg/l. The remaining procedure was as described in Experiment I. The data were shown in a graph and the moles of copper

bound per mole of sodium β-caseinate were read from the graph.

Experiment 5.--Binding of Copper with β-Lactoglobulin

The concentration of β-lactoglobulin solution used in this experiment was 1.420 mg/l. The rest of the procedure was as described in Experiment I. The data were represented in a graph and the moles of copper bound per mole of β-lactoglobulin were determined from the graph.

Experiment 6.--Lipid Oxidation in Model Systems of Copper-Micellar Caseinate, Washed Fat-Globules and Milk Dialyzate

The copper proteinate solution of micellar casein (3.5% V/V) was suspended in the model system of washed fat-globules and milk dialyzate. The model system without copper was specified as the control. The model systems with and without copper were given the heat treatments of 145°F for 30 minutes and 190°F for 10 minutes. After cooling, the samples were stored at 35°F for 10 days. At 2-, 4-, 6-, 8-, and 10-day intervals, oxidation was followed by TBA and peroxide value. Data were shown in graphs by plotting the TBA peroxide values against the storage time.

Experiment 7.--Lipid Oxidation in Model Systems of Copper-Fat-Globule Membrane Proteinate, Washed Fat-Globules and Milk Dialyzate

The preceding experiment was repeated with the exception that the fat-globule membrane protein was substituted for micellar casein.

Experiment 8.--Lipid Oxidation in Model Systems of Copper Sodium α-Caseinate, Washed Fat-Globules and Milk Dialyzate

The copper proteinate of sodium α-caseinate was suspended in the model system of washed fat-globules and milk dialyzate. The remaining procedure was similar to that of Experiment 6.

Experiment 9.--Lipid Oxidation in Model Systems of Copper Sodium β-Caseinate, Washed Fat-Globules and Milk Dialyzate

The copper proteinate of sodium β-caseinate was suspended in the model system of washed fat-globules and milk dialyzate. The rest of the procedure was as described in Experiment 6.

Experiment 10.--Lipid Oxidation in Model Systems of Copper-β-Lactoglobulin Proteinate, Washed Fat-Globules and Milk Dialyzate

The copper proteinate of β-lactoglobulin was suspended in the model system of washed fat-globules and milk dialyzate. The remaining procedure was similar to that of Experiment 6.

Experiment 11.--Lipid Oxidation in Heated and Unheated Model Systems of Copper-β-Lactoglobulin Proteinate, Washed Fat-Globules and Milk Dialyzate

Model systems of β-lactoglobulin, washed fat-globules and milk dialyzate were heated separately at 145°F for 30 minutes and 190°F for 10 minutes. After cooling, the model system of copper proteinate of β-lactoglobulin (3.5% V/V) was suspended in a model system of washed fat-globules and milk dialyzate. An unheated model system of copper proteinate of β-lactoglobulin was also incorporated into an unheated model system of washed fat-globules and milk dialyzate. The samples were stored at 35°F for 10 days. At 2-, 4-, 6-, 8-, and 10-day intervals, oxidation was studied by running TBA and peroxide value tests.

Experiment 12.--Lipid Oxidation in Heated and Unheated Model Systems of Copper-Fat-Globule-Membrane Proteinate, Washed Fat-Globules and Milk Dialyzate

The procedure of the preceding experiment was repeated with the exception that the fat-globule membrane protein was substituted for β-lactoglobulin.

RESULTS AND DISCUSSION

The copper-protein complex catalyzes lipid oxidation in fluid milk systems. The influence of copper-protein ratio on lipid autoxidation may account for some inconsistent statements in the literature regarding the catalytic activity of copper when in combination with proteins. In experiments with a model system of linoleate, addition of 10^{-4} M albumin decreased the catalytic activity of 10^{-4} M copper in the presence of ascorbic acid (98). Lea (70) used copper concentrations of 0.4 x 10^{-6} to 2.4 x 10^{-6} M with 6 x 10^{-4} M albumin (4%) and found that at these high concentrations of copper, the copper catalysis of fat oxidation was inhibited. In contrast, Tappel (108) and Jenness and Patton (50) considered that the combination of copper with protein increased the catalytic activity of copper in milk. In view of these somewhat conflicting reports, and also because of precise data regarding the exact amounts of copper bound by individual milk proteins were lacking, appropriate experiments were designed to determine the binding of copper with micellar casein, fat-globule membrane protein, sodium α-casein, sodium β-casein and β-lactoglobulin.

The extent of binding of copper by the above-mentioned proteins is illustrated in Figures 1 to 5. The degree of binding is represented in graphs of the average number of moles of copper bound per mole protein versus the logarithm of the concentration of unbound copper. These data were obtained by the technique of equilibrium dialysis at a pH of 6.5 to approximate conditions of hydronium ion concentration in cow's milk. The number of moles of copper bound per mole of micellar casein is shown in Figure 1. One mole of micellar casein bound 67 moles of copper. In order to compare relative binding capacities, the number of moles of copper bound per mole of protein was determined at a free copper concentration of 1×10^{-5} M which is equivalent to 3 ppm of copper ion surrounding the dialysis bag following equilibrium for 72 hours. The number of moles of copper bound per mole of fat-globule membrane protein is presented in Figure 2. One mole of fat-globule membrane bound 24 moles of copper. The number of moles of copper bound per mole of sodium α-caseinate is given in Figure 3. One mole of sodium α-caseinate bound 10 moles of copper. The number of moles of copper bound per mole of sodium β-caseinate is depicted in Figure 4. One mole of sodium β-caseinate bound two moles of copper. The number of moles of copper bound per mole of β-lactoglobulin is shown in Figure 5. One mole of β-lactoglobulin bound 2.5 moles of copper.

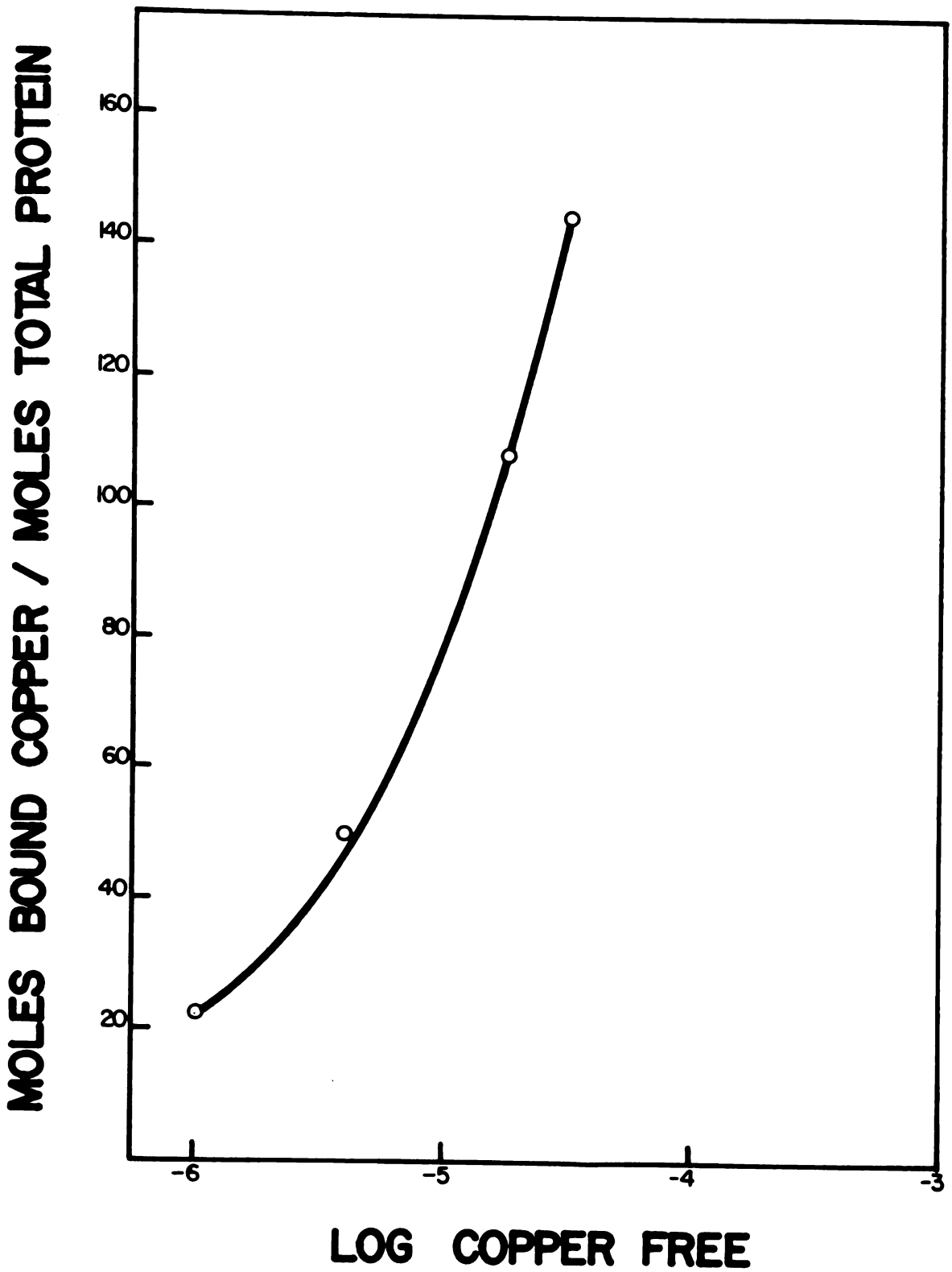

Figure 1. Binding of copper with micellar casein.

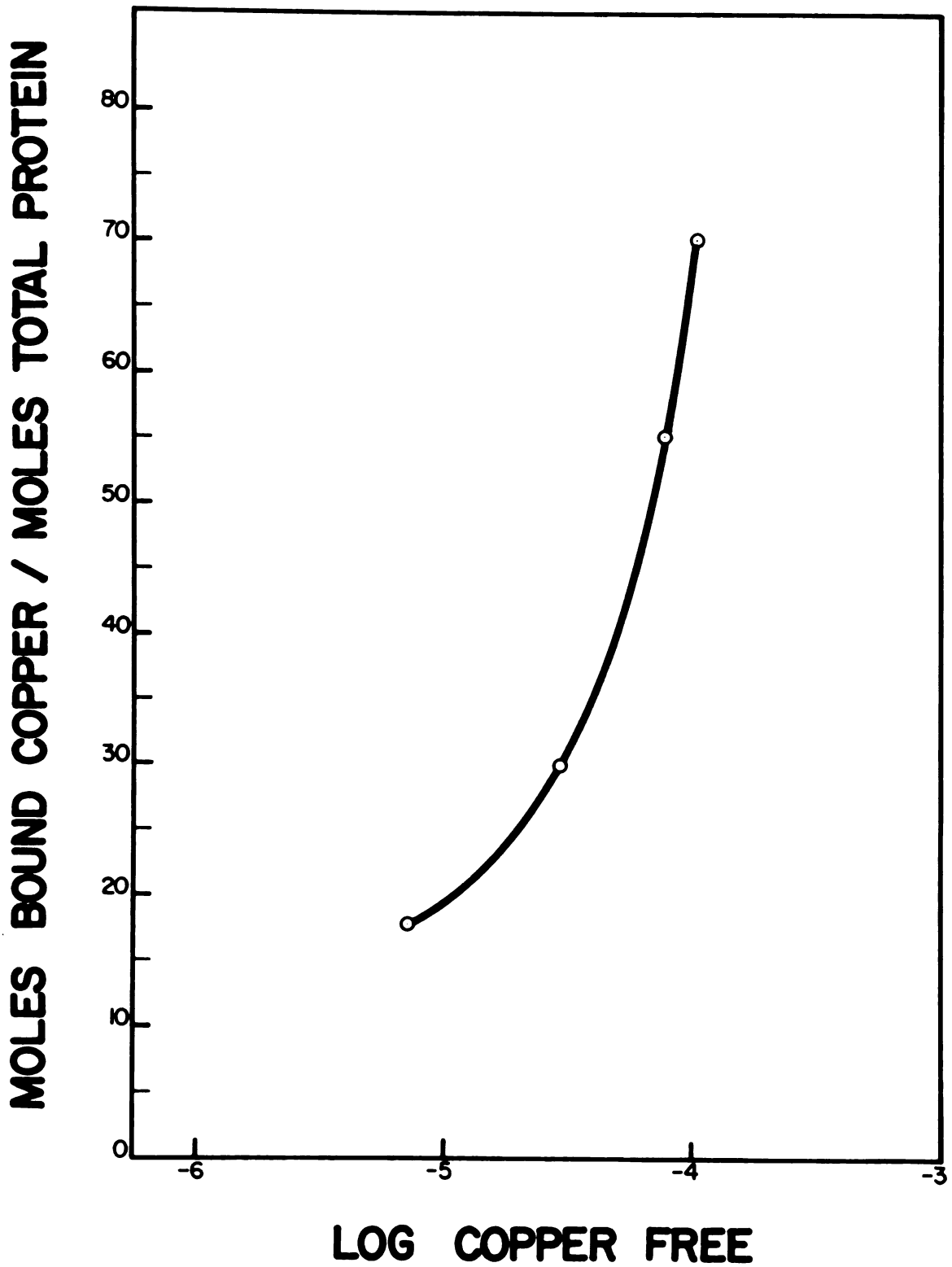

Figure 2. Binding of copper with fat-globule membrane protein.

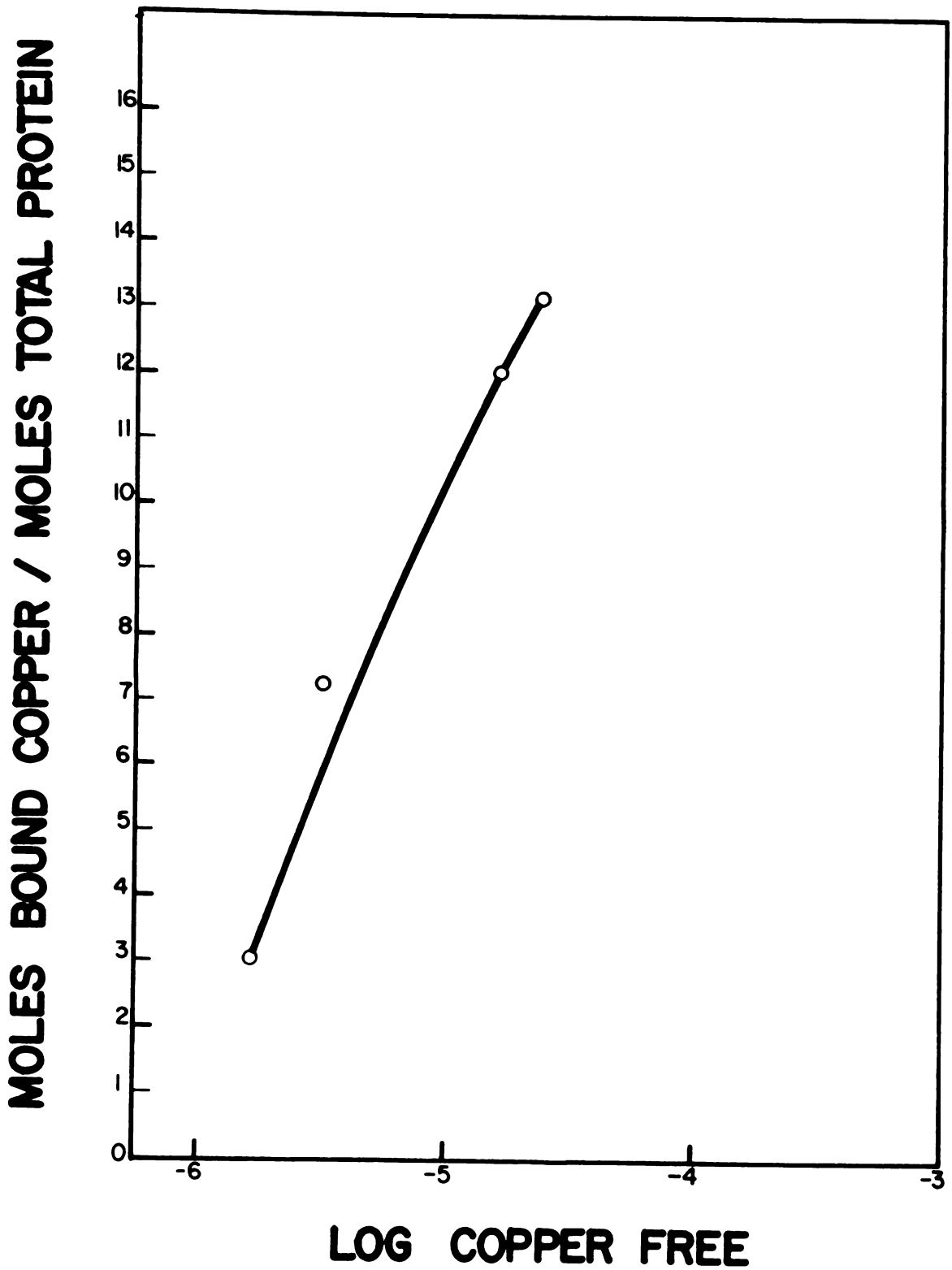

Figure 3. Binding of copper with sodium α-caseinate.

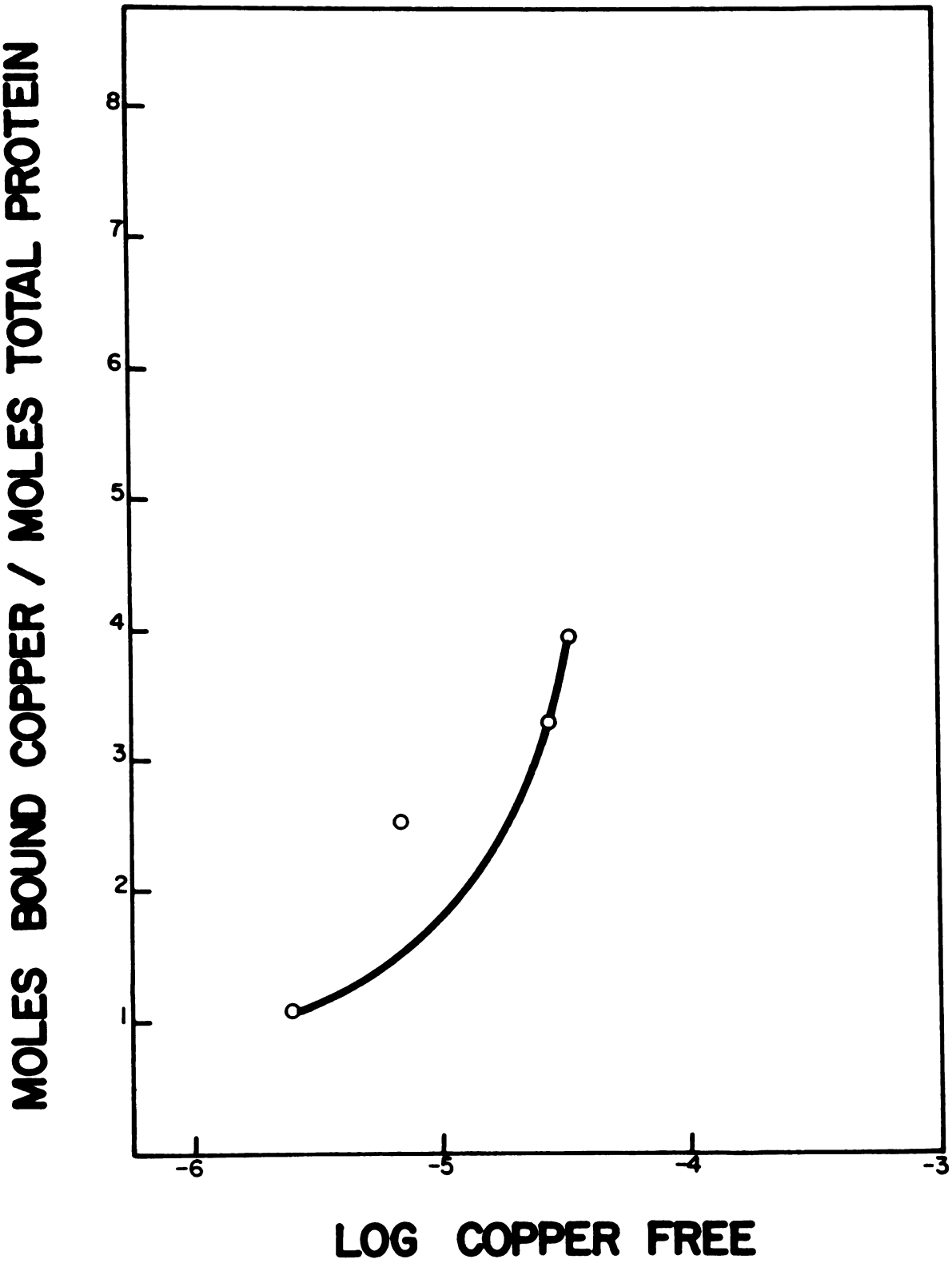

Figure 4. Binding of copper with sodium β-caseinate.

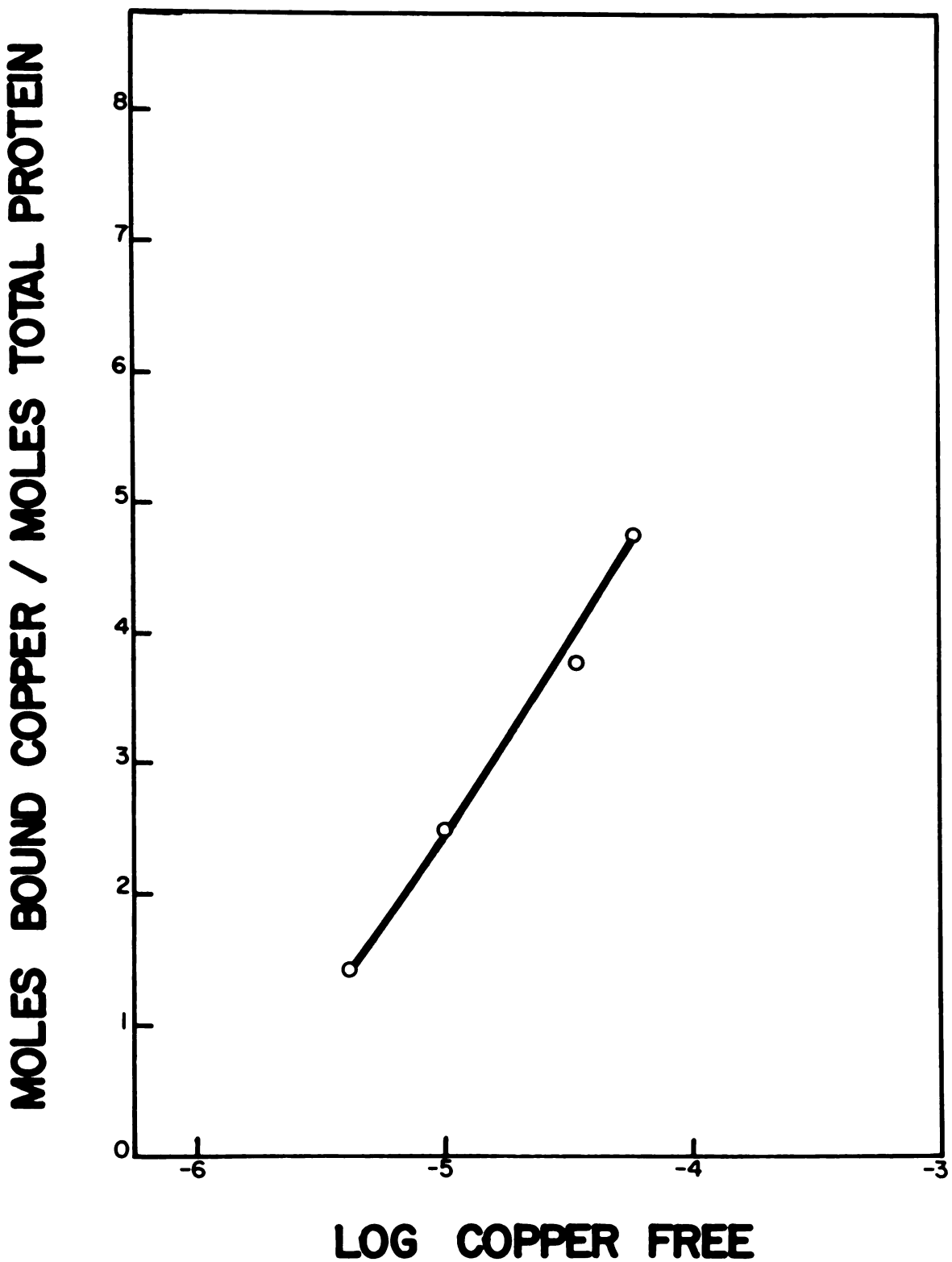

Figure 5. Binding of copper with β-lactoglobulin.

The results clearly demonstrate the great difference in ability of certain milk proteins to bind copper ions. Micellar casein, obtained by high speed centrifugation of skimmilk bound the greatest amount of copper. The fat-globule membrane protein, which of course, is intimately associated with the lipids of the fat-globule, demonstrated the second greatest affinity for copper ions. Much lesser amounts of copper were bound by other proteins studied: sodium α-caseinate, β-lactoglobulin and sodium β-caseinate. These results agree with the observation of King et al (54) who observed that most of the added copper (75%) was associated with the casein fraction. King and William (53) later on also reported that the copper binding activity of the fat-globule membrane was greater than that of some other copper-binding substances in skimmilk. This seems to be consistent with the much greater concentration of natural copper in combination with the fat-globule membrane material as compared to the other protein-containing fractions of milk. Koops (62) showed that affinity of the proteins to copper at pH 6.8 was decreased in the following order: fat-globule membrane protein, sodium caseinate, β-lactalbumin and β-lactoglobulin. According to Fiess and Klotz (30), the affinity of the proteins to copper at pH 6.5 was found to decrease in the following sequence: α-casein, β-casein, bovine serum albumin, β-lactoglobulin and

lysozyme. Menger and Mulder (73) reported that added copper was primarily associated with skimmilk proteins. Herald et al. (44) found that many of the trace elements (copper, iron, zinc, silver, etc.) reported in the whole milk were concentrated on the fat-globule membrane possibly as constituent elements of various metabolic complexes. Samuelsson (93) reported that copper is associated to a certain extent with the fat-globule membrane protein, but most copper was found in the serum. This implies that the substances which accompany the fat, namely the membrane proteins have a great capacity for binding copper. Certainly this may well be important in the oxidation of milk as the phospholipids are present on the surface of the fat-globule.

The beneficial effect of heating fluid milk in excess of temperatures normally used in pasteurization on the resistance of the resulting dry whole milk to the development of tallowy flavor was first demonstrated by Holm et al. (49) in 1926. This observation has been corroborated by many other workers in the field. Later Gould and Sommer (35) found a relationship between high heat treatment and the appearance of cooked flavor when copper was added before heat treatment. Further, their study showed hydrogen sulfide to be correlated with the cooked flavor and to be involved in the heat retardation of the development of the oxidized flavor. These findings

have been verified by the work of Josephson and Doan (51). Greenbank (36) reported that the thermal inhibition of oxidized flavor acts through a lowering of the oxidation reduction potential. This has been confirmed by both the work of Gould and Sommer (35) and Josephson and Doan (51) who found that the sulfide compounds, liberated when a cooked flavor develops, lowered the potential of milk. According to Thomas (110) the heat-liberated sulfhydryl groups presumably function as an antioxidant in the liquid system through preferential oxidation to some undetermined product.

In order to determine the effect of low and high heat treatments on various copper-protein complexes suspended in a model system of washed fat-globules and milk dialyzate, a series of experiments was conducted. A model system of washed fat-globules and milk dialyzate was used in all of these experiments because the use of such model system will overcome some of the inherent problems associated with the complexity of milk. The extent of lipid oxidation in the model systems of copper micellar caseinate, washed fat-globules and milk dialyzate is shown in Figures 6a and 6b. Maximum TBA values corresponding to an optical density of 0.32 and peroxide values of 11.0 milliequivalent of oxygen per kg of fat were obtained in this experiment. Results presented in Figure 6a indicate that the unheated model systems containing copper micellar caseinate and micellar casein

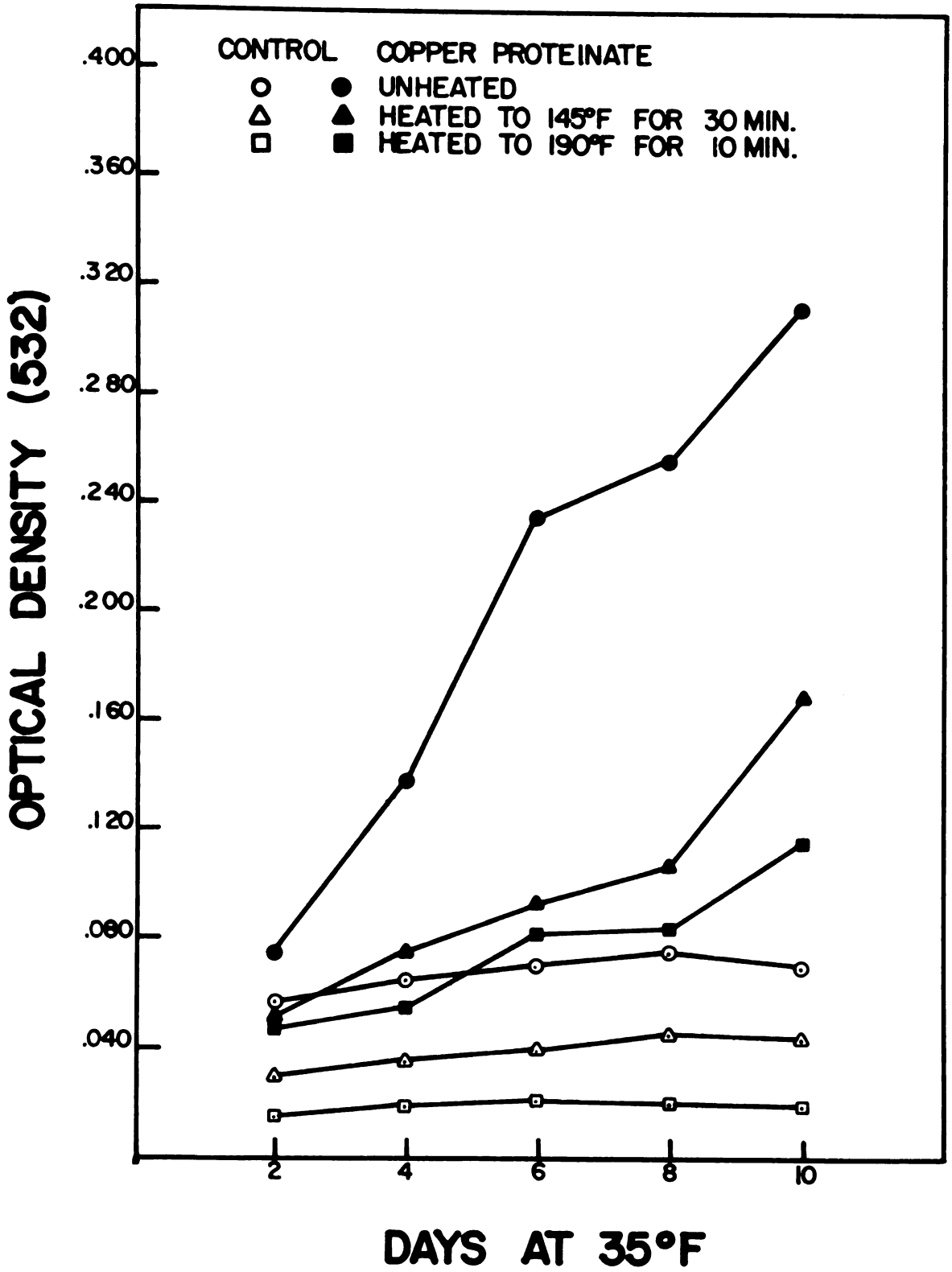

Figure 6a. Lipid oxidation in model systems of copper-micellar caseinate, washed fat-globules and milk dialyzate (TBA).

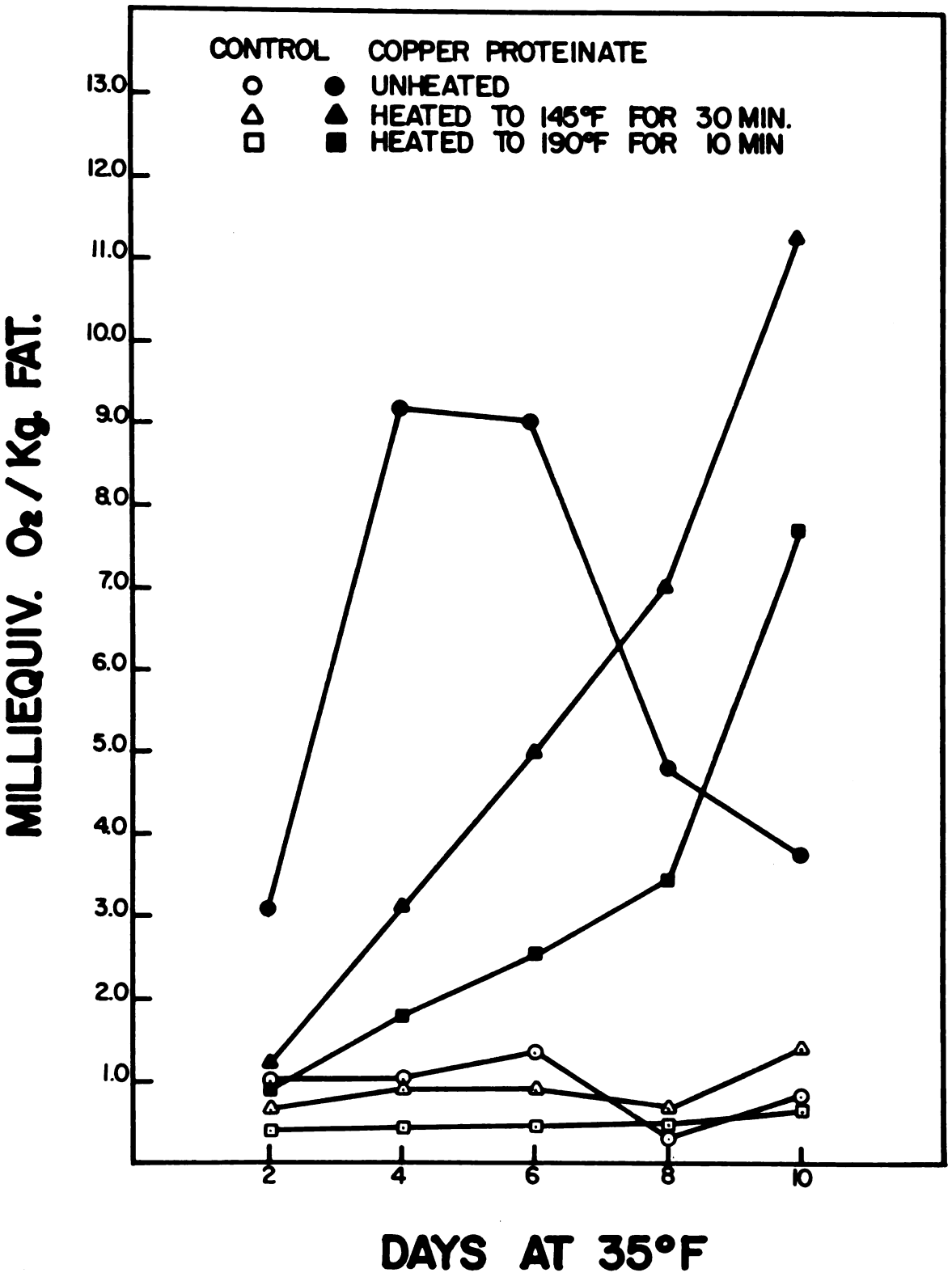

Figure 6b. Lipid oxidation in model systems of copper-micellar caseinate, washed fat-globules and milk dialyzate (P.V.).

exhibited the greatest amount of oxidation of the six model systems. The systems heated to 145°F for 30 minutes showed less oxidation than unheated systems. The systems heated to 190°F for 10 minutes demonstrated the least amount of oxidation compared to unheated systems. The extent of oxidation in the model system containing copper micellar caseinate increased considerably with the increase of storage period. The maximum increase in oxidation in such systems was observed after 10 days storage. This of course is similar to the pattern of oxidation which occurs in normal whole cow's milk when copper is present. Milk containing copper in a quantity sufficient to catalyze lipid autoxidation (.05 - 5.0 ppm) will invariably be objectionably oxidized within 10 days storage at 35-40°F. The systems subjected to both low and high heat treatments exhibited significant amount of oxidation after 10 days of the storage period. Model systems containing only micellar casein showed very slight oxidation during the storage period of 10 days.

Results presented in Figure 6b demonstrate that peroxide values for unheated model system containing copper micellar casein showed an abrupt fall after the 6th day of storage. Similar results were obtained from unheated model systems which contained only micellar casein. However, the heated systems with and without copper did not show a similar fall in the peroxide values.

Wilkinson (120) reported a similar phenomenon. According to him the relationship of peroxide value to off-flavor does not always hold well in dairy products in which oxidation has been catalyzed by added copper, the peroxide value often being lower than that of control samples to which no copper has been added. Ritter and Nussbaumer (89) found that copper added to butter-fat accelerated peroxide formation in the presence of light. Later they reported (90) that small amounts of copper palmitate caused a large increase of peroxide value, but large amounts induced little or no change. The reason for this phenomenon is that copper not only accelerates the assimilation of oxygen to increase the peroxide number, but also hastens the further decomposition of the fat peroxides to free radicals. This suggests why the peroxide number may decrease in the presence of greater amounts of copper and why this value taken at a given time in a stored sample is not an infallible index of acceptability.

Results of lipid oxidation in model systems of copper-fat-globule membrane proteinate, washed fat-globules and milk dialyzate are shown in Figures 7a and 7b. Maximum TBA values of .115 optical density and peroxide values of 3.78 milliequivalents of oxygen per kg of fat were obtained in this experiment. The results presented in Figure 7a are similar to those shown in

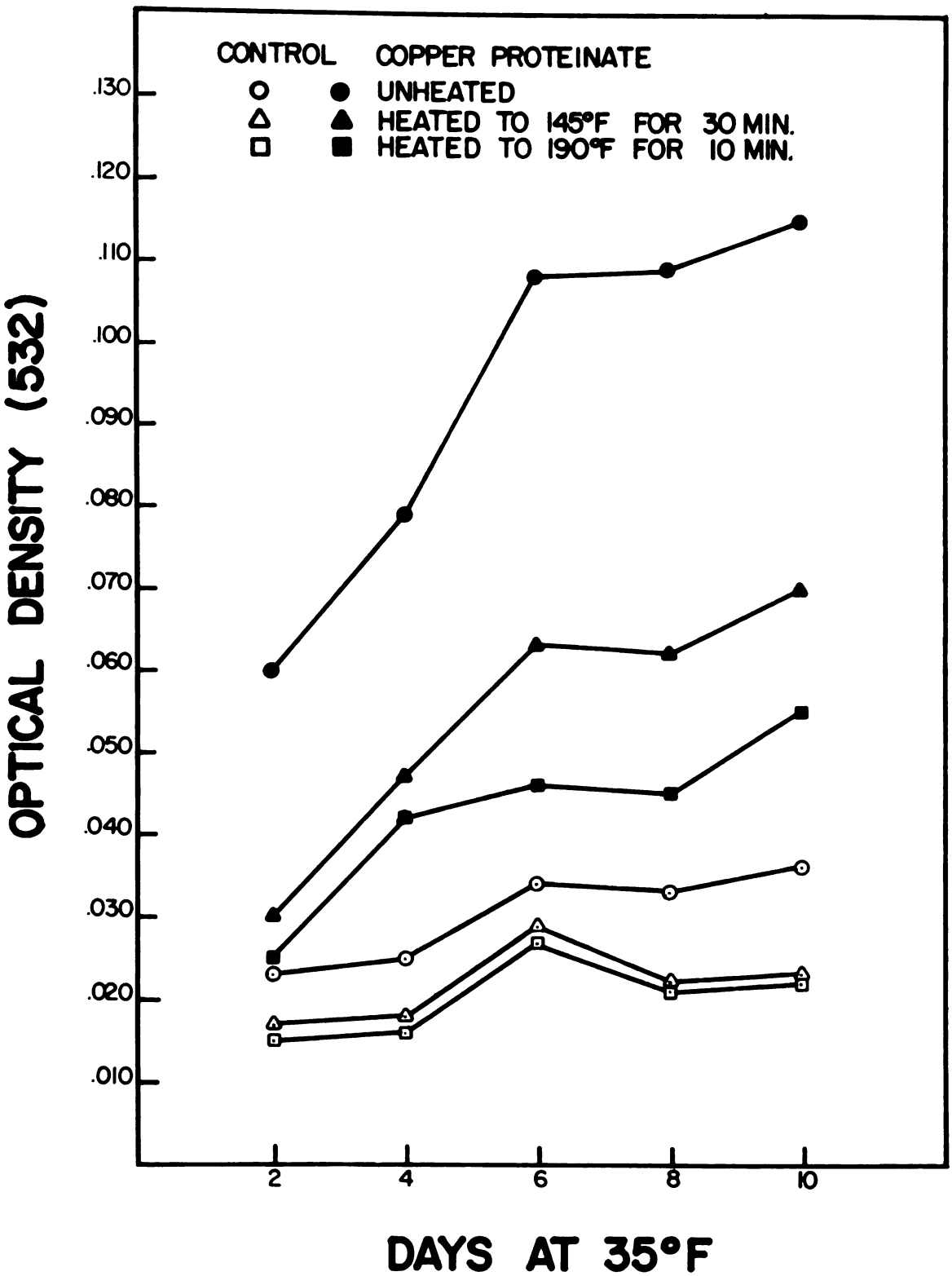

Figure 7a. Lipid oxidation in model systems of copper-fat-globule membrane proteinate, washed fat-globules and milk dialyzate (TBA).

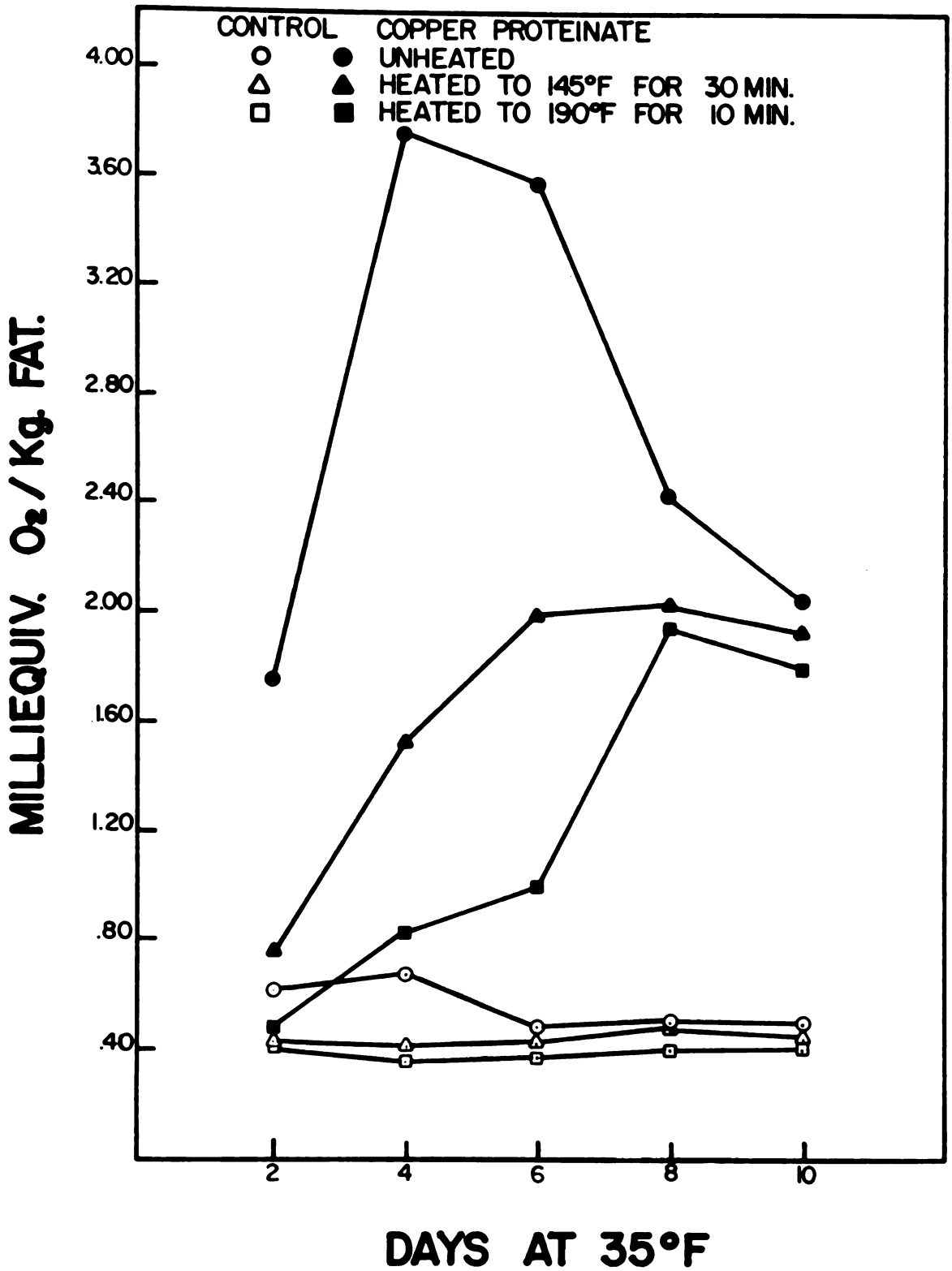

Figure 7b. Lipid oxidation in model systems of copper-fat-globule membrane proteinate washed fat-globules and milk dialyzate (P.V.).

Figure 6a. However, in unheated model systems containing copper-fat-globule membrane proteinate, the oxidation was significantly lower than those containing copper micellar caseinate. Both low and high heat treatments inhibited the extent of oxidation. Model systems subjected to high heat treatment indicated the least amount of oxidation. Control model systems heated to 145°F for 30 minutes and to 190°F for 10 minutes did not show any considerable difference in the intensity of oxidation. The extent of oxidation increased with increasing time of storage in the model systems containing copper-fat-globule membrane protein. In unheated and heated control model systems, the extent of oxidation accelerated up to the 6th day in storage; thereafter a decline was noted. Results shown in Figure 7b demonstrate that peroxide values for unheated model systems containing copper-fat-globule membrane proteinate agree closely with those containing copper micellar caseinate (Figure 6b). The curves for peroxide values obtained from heated systems containing copper-fat-globule membrane proteinate are characterized by an initial linear portion, followed by a gradual decrease in slope, terminating in a rather abrupt leveling off at some maximum value. Peroxide values obtained from heated control model systems showed minimal changes throughout the period of storage.

Figure 8 indicates the results of lipid oxidation in model systems of copper sodium α-caseinate, washed

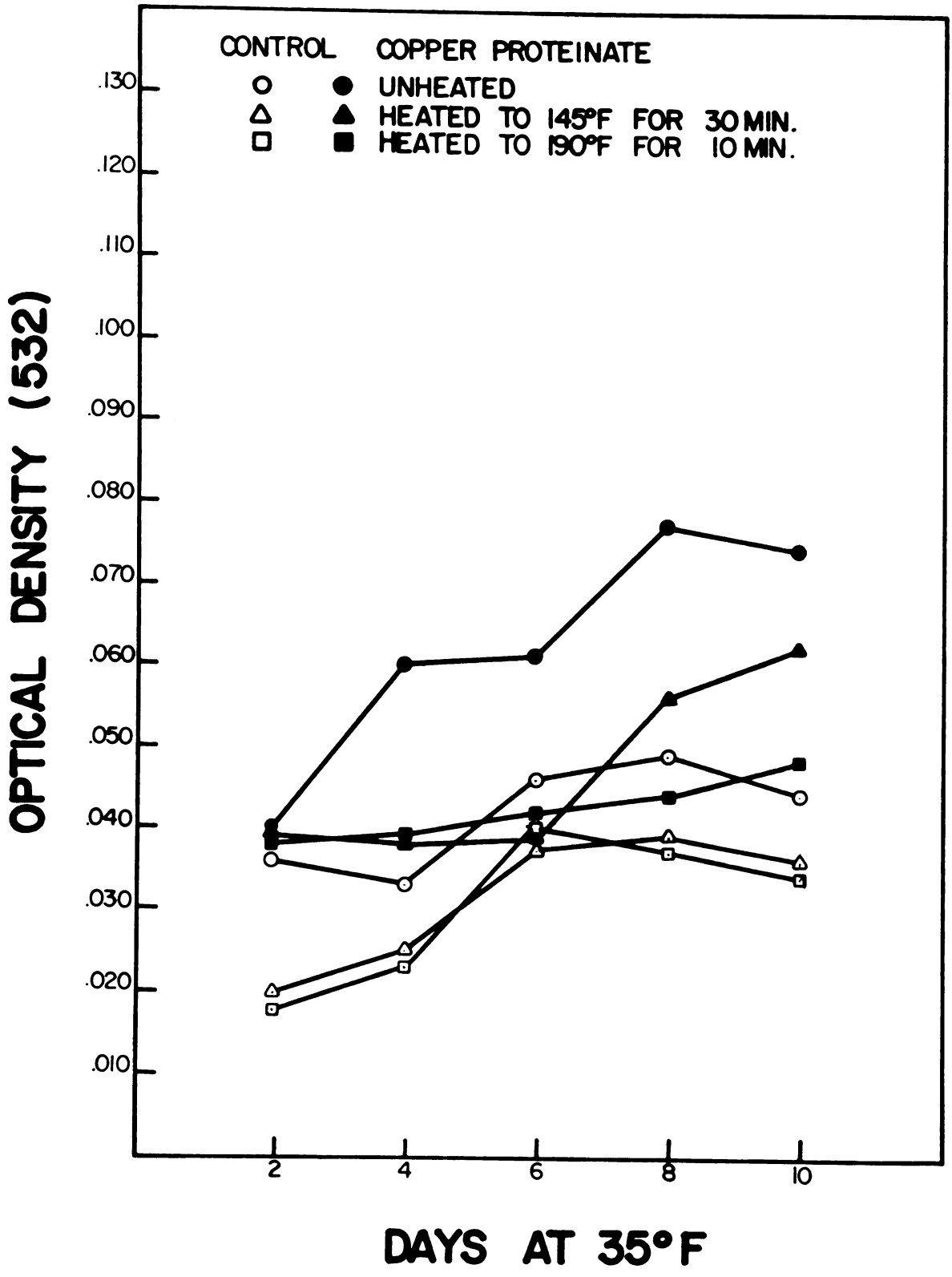

Figure 8. Lipid oxidation in model systems of copper sodium α-caseinate, washed fat-globules and milk dialyzate.

fat-globules and milk dialyzate. Maximum TBA values corresponding to an optical density of about 0.77 were obtained in this experiment. The data presented in Figure 8 are also somewhat similar to those shown in Figures 6a and 7a. The extent of oxidation in unheated model systems with and without copper increased up to 8th day in storage, afterwards the extent of oxidation decreased in intensity. The unheated model system containing copper sodium α-caseinate (Figure 8) was less strongly oxidized than systems containing copper micellar caseinate or copper-fat-globule membrane proteinate (Figures 6a and 7a). However, in systems containing no added copper the unheated model system containing sodium α-caseinate was more strongly oxidized than those containing micellar casein and fat-globule membrane protein. The low and high heat treatments caused a decrease in oxidation in model systems of sodium α-caseinate with and without copper. The extent of oxidation increased with increasing storage period in the heated model system containing copper sodium α-caseinate. In case of the heated model system containing sodium α-caseinate, the extent of oxidation increased up to 6th day of storage period, after which a decrease occurred.

Figure 9 shows the results of lipid oxidation in model systems of copper sodium β-caseinate, washed fat-globules and milk dialyzate. Maximum TBA values

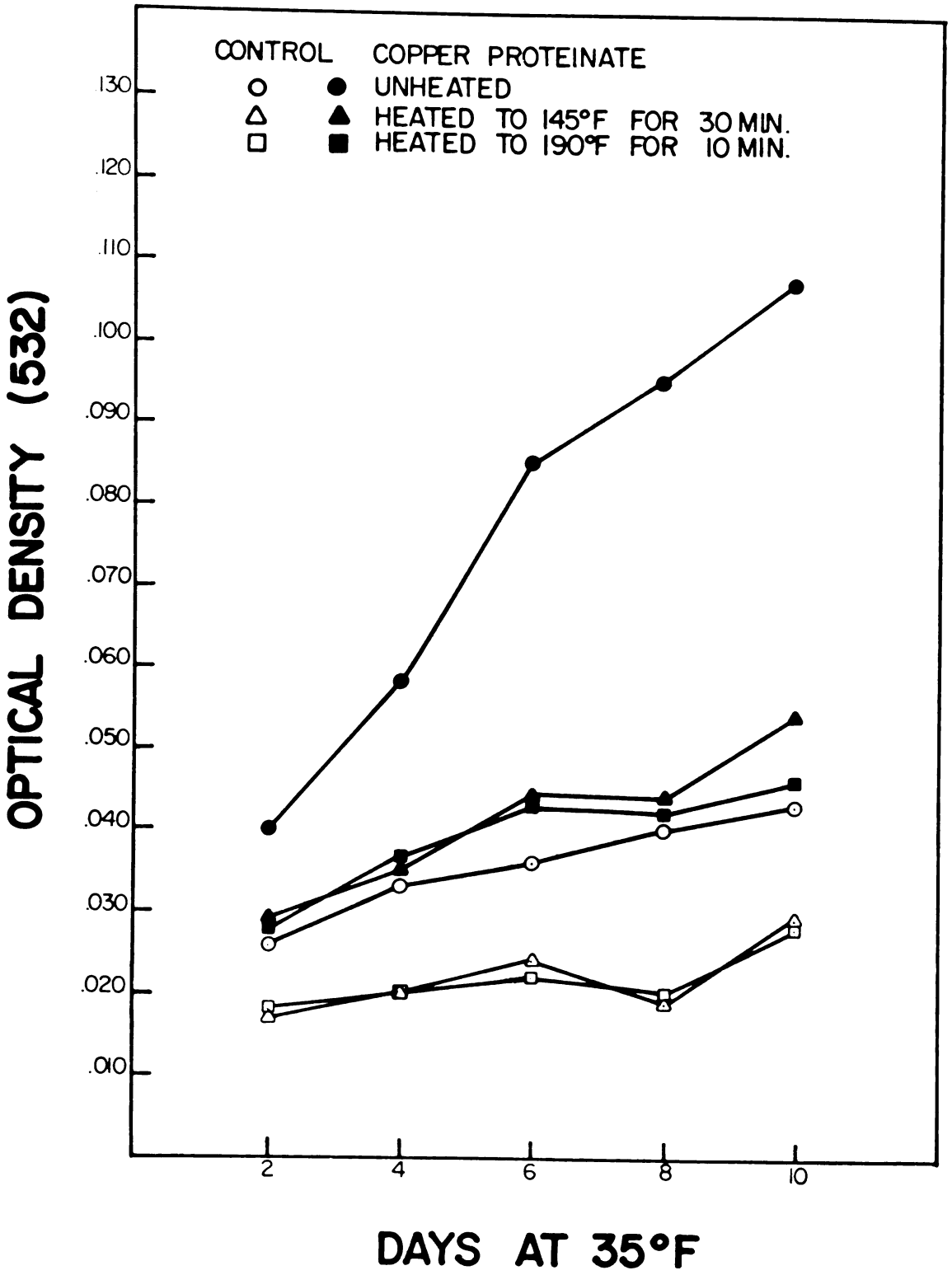

Figure 9. Lipid oxidation in model systems of copper sodium β-caseinate, washed fat-globules and milk dialyzate.

corresponding to an optical density of about 0.107 were obtained in this experiment. The results shown in Figure 9 indicate some similarity to those presented in Figures 6a, 7a, and 8. The extent of oxidation was directly proportional to the storage period in unheated and heated model system with and without copper. Unheated model system containing copper-sodium β-caseinate (Figure 9) was less strongly oxidized than those containing copper micellar caseinate and copper-fat-globule membrane proteinate (Figures 6a and 7a). However the unheated model system containing copper sodium β-caseinate was somewhat more oxidized than that containing sodium α-caseinate (Figure 8). The high heat treatment caused the greatest decrease in oxidation in model system containing copper sodium β-caseinate. As shown by the curves in Figure 9, the effect of low and high heat treatments on the lipid oxidation in model systems without copper was over-lapping. The over-lapping effects of high and low heat treatments varied with the storage period of the samples.

Possibly the lessened effects of heat on the model systems of control (containing no added copper) α- and β-caseinate are due to the fact that these proteins as isolated and prepared are not typical of their natural state in milk. They have in effect been fractionated from the much larger natural casein micelle by treatment with urea and their binding affinities are much lower. Thus, when resuspended in the milk dialyzate, they now

exist more or less as a synthetic entity and are less effective in binding metals or functioning as catalysts.

The results of lipid oxidation in model systems of copper β-lactoglobulin, washed fat-globules and milk dialyzate are demonstrated in Figure 10. Maximum TBA values corresponding to an optical density of about 0.92 were obtained in this experiment. The results presented in Figure 10 are similar to those shown in Figures 6a, 7a, 8, and 9. The extent of oxidation was directly proportional to the storage period in heated and unheated model systems with and without copper. The unheated model system containing copper β-lactoglobulin proteinate (Figure 10) was less strongly oxidized than those containing copper micellar caseinate, copper-fat-globule membrane proteinate and copper-sodium β-caseinate (Figures 6a, 7a, and 9). The unheated model system containing copper-β-lactoglobulin was more strongly oxidized than that containing copper-sodium α-caseinate. Both low and high heat treatments decreased the extent of oxidation in model systems containing copper-β-lactoglobulin proteinate. The high heat treatment caused the greatest amount of decrease in the extent of oxidation in model systems with and without copper.

The data presented (Figures 6a, 6b, 7a, 7b, 8, 9, and 10) thus far have indicated that unheated model systems containing copper micellar caseinate and copper-fat-globule membrane proteinate exhibited the greatest

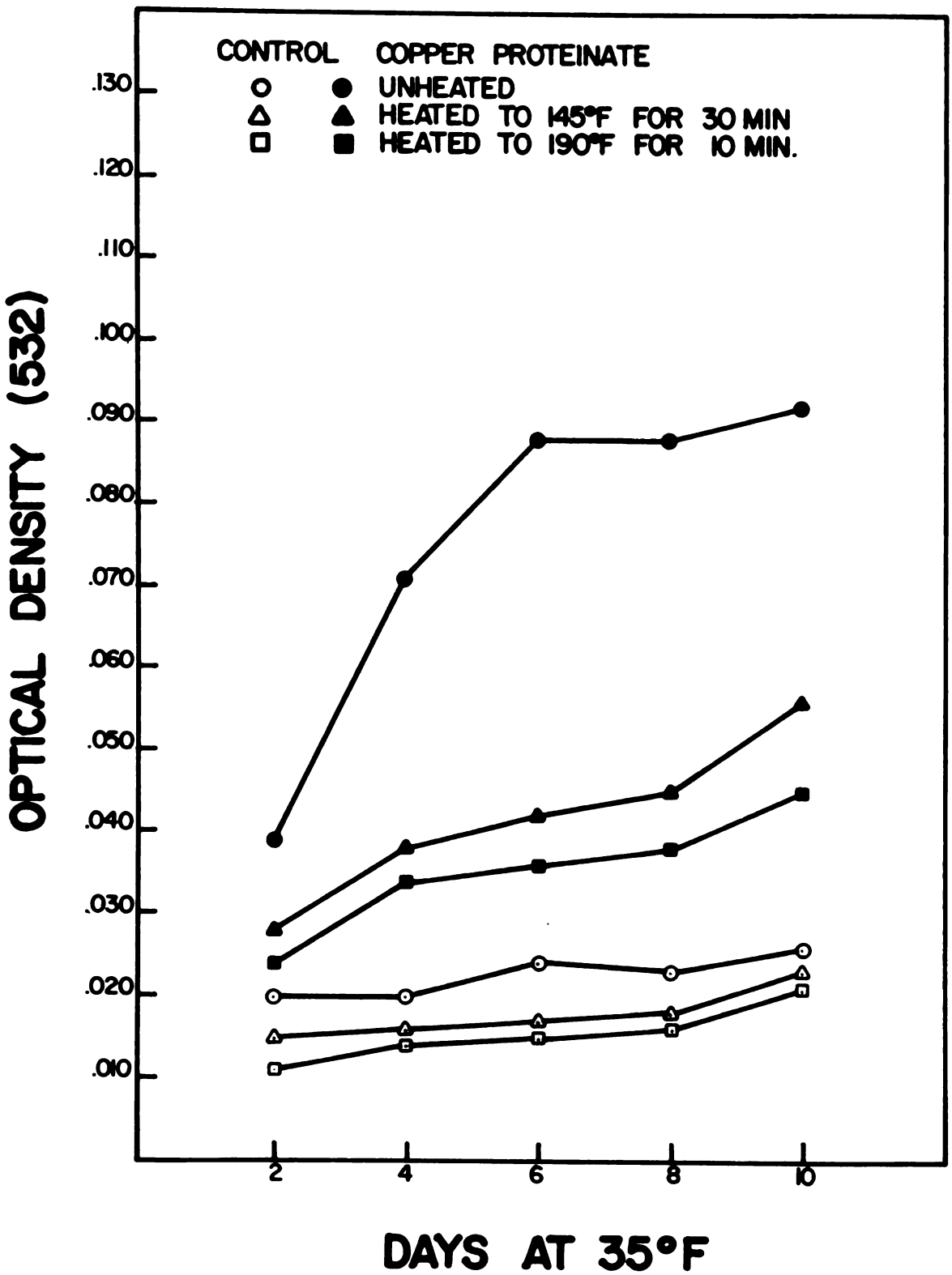

Figure 10. Lipid oxidation in model systems of copper-β-lactoglobulin proteinate, washed fat-globules and milk dialyzate.

amount of oxidation in their systems. This was attributed to greater copper binding capacity of micellar casein and fat-globule membrane protein. The results obtained from experiments involving lipid oxidation in model systems clearly show that the model systems subjected to low and high heat treatments exhibited less tendency to oxidize than unheated controls. The model system subjected to the highest heat treatment demonstrated the least amount of oxidation. The decrease in the extent of oxidation suggests that heating of copper proteinates, washed fat-globules and milk dialyzate at 145°F for 30 minutes causes the formation of reducing substances. The reducing substances in turn reduce the pro-oxidant activity of copper and lessen the extent of oxidation in the model systems. Heating of above systems at 190°F for 10 minutes results in the formation of greater quantities of reducing substances, probably the most important of which are sulfhydryl groups. The heat-activated sulfhydryl groups may complex with copper (as does mercury to form mercaptides) during high heat treatment and bring about further reduction in the pro-oxidant properties of copper. Systems of copper proteinates, washed fat-globules and milk dialyzate heated at 145°F for 30 minutes would of course show much lower levels of heat activation. Similar observations have been demonstrated by several workers (41) who reported that high

temperature, short time heat treatments above 212°F imparts greater reducing properties to fluid milk than holder treatment at lower (145°F) temperatures.

It is quite obvious from examination of the data in this investigation and also from the literature already published on the subject, that heating of fluid milk induces an ill defined change in the structure of some of the milk components with the result that milk fat becomes more resistant to oxidation. Radema (86) has demonstrated that heating high fat milk or cream to 85°C or higher for periods up to 30 minutes brings about a shift in some of the phospholipid material from the fat-globule surface. This indicates a definite alteration of the fat-globule surface upon high heat treatment. It is recognized that significant amounts of copper and iron in milk are combined with the fat-globule membrane or are absorbed on the fat-globule surface as a metal protein complex, and the ionic concentrations of these metals is extremely low. Mulder and Koppeljan's data (77), and also this research, show that concentration of copper at the fat globule interface may be quite high. This proximity of copper and fat in milk may enable the copper to function effectively as a catalyst in lipid oxidation. It is probable that the effect of heat on milk may be the displacement of copper from the fat globule surface to a position or configuration where it would be less able to catalyse

oxidation. There is also a possibility that heat may alter the metal in denatured protein particles and make it less active as a catalyst.

Copper is a well-known catalyst of oxidized flavor in milk and dairy products. Tappel (108) has reported that "catalysis of unsaturated fat oxidation by copper proteins may be the cause of oxidative rancidity in butter and other dairy products." Virtually no ionic copper is present in milk; however, both copper and iron catalyze the oxidation of fat in dairy products. Dills and Nelson (22) isolated a copper-bearing protein from cow's milk and found that the copper in the protein was non-ionic and could not be removed by dialysis at pH 6.5. Copper-proteins formed by the binding of copper ions to conalbumin, serum albumin or caseinate appear to be more effective catalysts for linoleate oxidation than copper alone. The increase in oxidation of linoleate when catalyzed by copper proteins as compared to the catalytic effect of ionic copper is attributed to increased ease of formation and increased stability of the intermediate complex of linoleate peroxide-copper-protein (108). According to Tappel (108), copper proteins probably catalyze the oxidation of linoleate by reacting with linoleate peroxides and catalyzing their decomposition into chain-initiating free radicals. The overall reaction might be represented as follows:

$$Cu^{++} - \text{protein} + ROOH \longrightarrow ROO^{-} - Cu^{++} - \text{protein}$$

$$\longrightarrow R\overset{*}{O} + \overset{*}{O}H + Cu^{++} - \text{protein}$$

In the present investigation, copper-proteins formed by the binding of copper to micellar casein, fat-globule membrane protein, sodium α-casein, sodium β-casein, and β-lactoglobulin seem to be strong catalysts for lipid oxidation in the model system of washed fat-globules and milk dialyzate.

Additional experiments were carried out in order to find the effect of low and high heat treatments on lipid oxidation in model systems (copper proteinate, and washed fat-globules and milk dialyzate) in which each of these components was individually heated. The heated components were then combined as a model system. The intent of these experiments was to determine whether any alteration in proxidant activity of copper-protein occurred when the constituents of the model system were heated separately. Complex interactions could conceivably occur between metal protein and lipo-protein when an entire model system was heated. Such interactions would be minimized if constituents were individually heated and then combined.

The results of lipid oxidation in heated and unheated model systems of copper β-lactoglobulin, washed fat-globules and milk dialyzate are shown in Figures 11a and 11b. The data presented in Figure 11a were quite

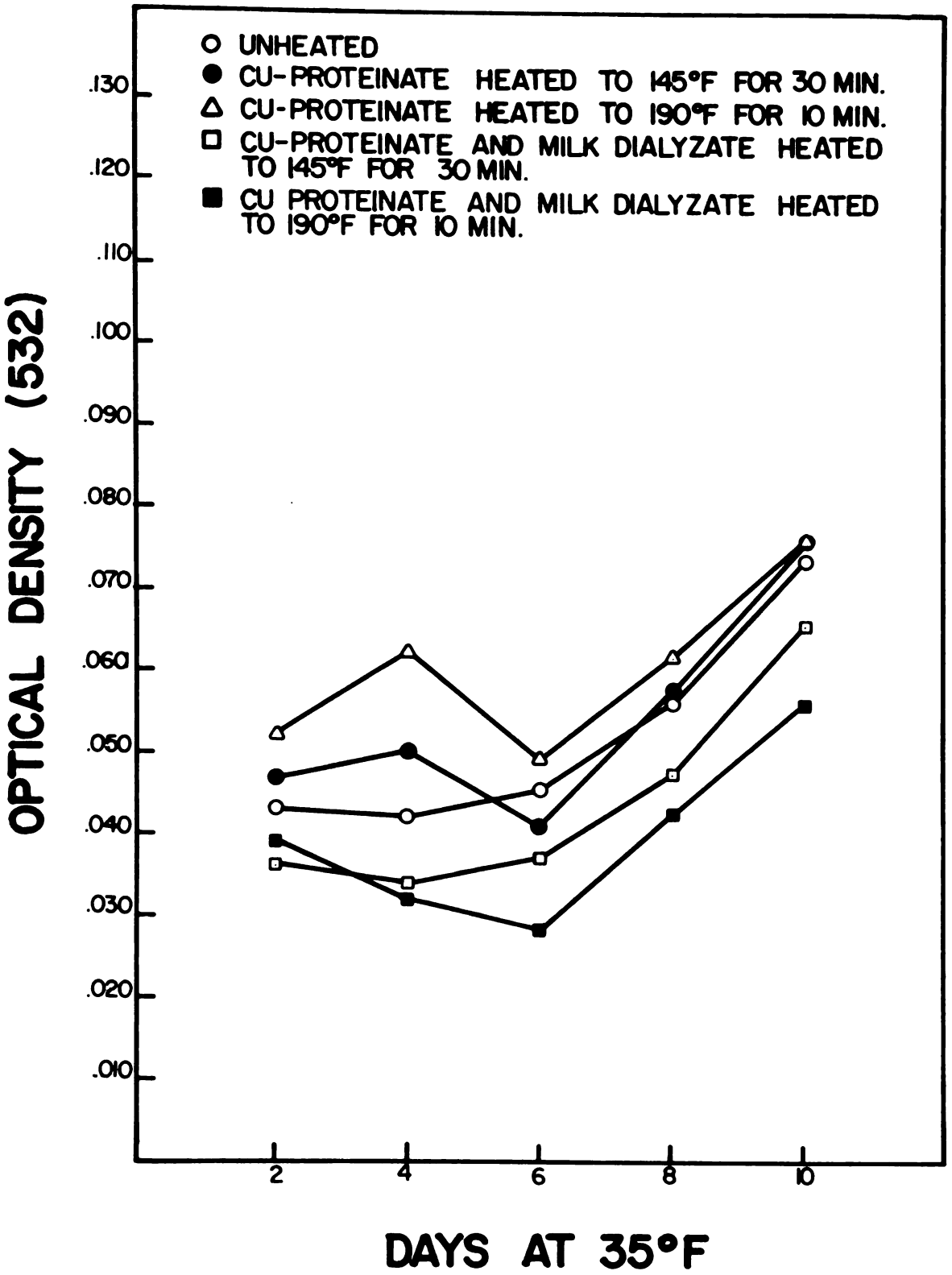

Figure 11a. Lipid oxidation in heated and unheated model systems of copper-β-lactoglobulin proteinate, washed fat-globules and milk dialyzate (TBA).

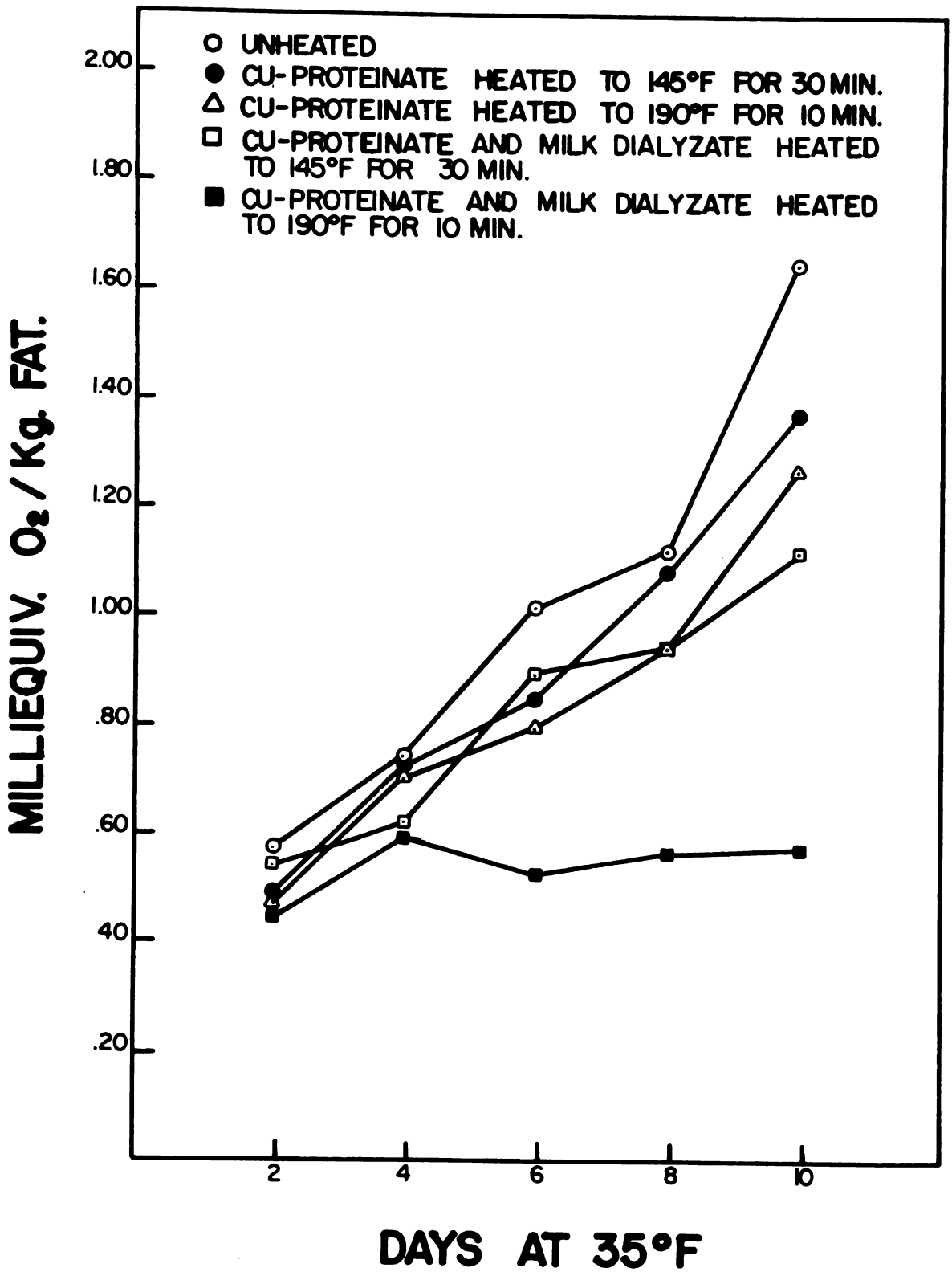

Figure 11b. Lipid oxidation in heated and unheated model systems of copper-β-lactoglobulin proteinate, washed fat-globules and milk dialyzate (P.V.).

different from those shown previously in Figures 6a, 7a, 8, 9, and 10. The results shown here (Figure 11a) on the basis of TBA values indicated that unheated model systems demonstrated lesser amounts of oxidation than those containing heated (low and high temperature) copper proteinate. A maximum decrease in the oxidation was found in the model systems of copper β-lactoglobulin, washed fat-globules and milk dialyzate when they were heated separately to 190°F for 10 minutes and then combined. The results shown in Figure 11b indicated from peroxide values that the unheated model systems were more strongly oxidized than those subjected to low and high heat treatments. When both copper proteinate, and the model system of washed fat-globules and milk dialyzate were heated individually to 190°F for 10 minutes, the extent of oxidation was decreased. Such heated systems did not show any significant increase in the extent of oxidation with increasing storage period.

Figures 12a and 12b indicated the results of lipid oxidation in heated and unheated model systems of copper-fat-globule membrane proteinate, washed fat-globules and milk dialyzate. Most of the results presented in Figure 12a were similar to those shown in Figure 11a. However, higher TBA values were obtained (Figure 12a) from model systems containing heated copper proteinate than those shown in Figure 11a. The higher TBA values could be related to higher amount of oxidation in these systems.

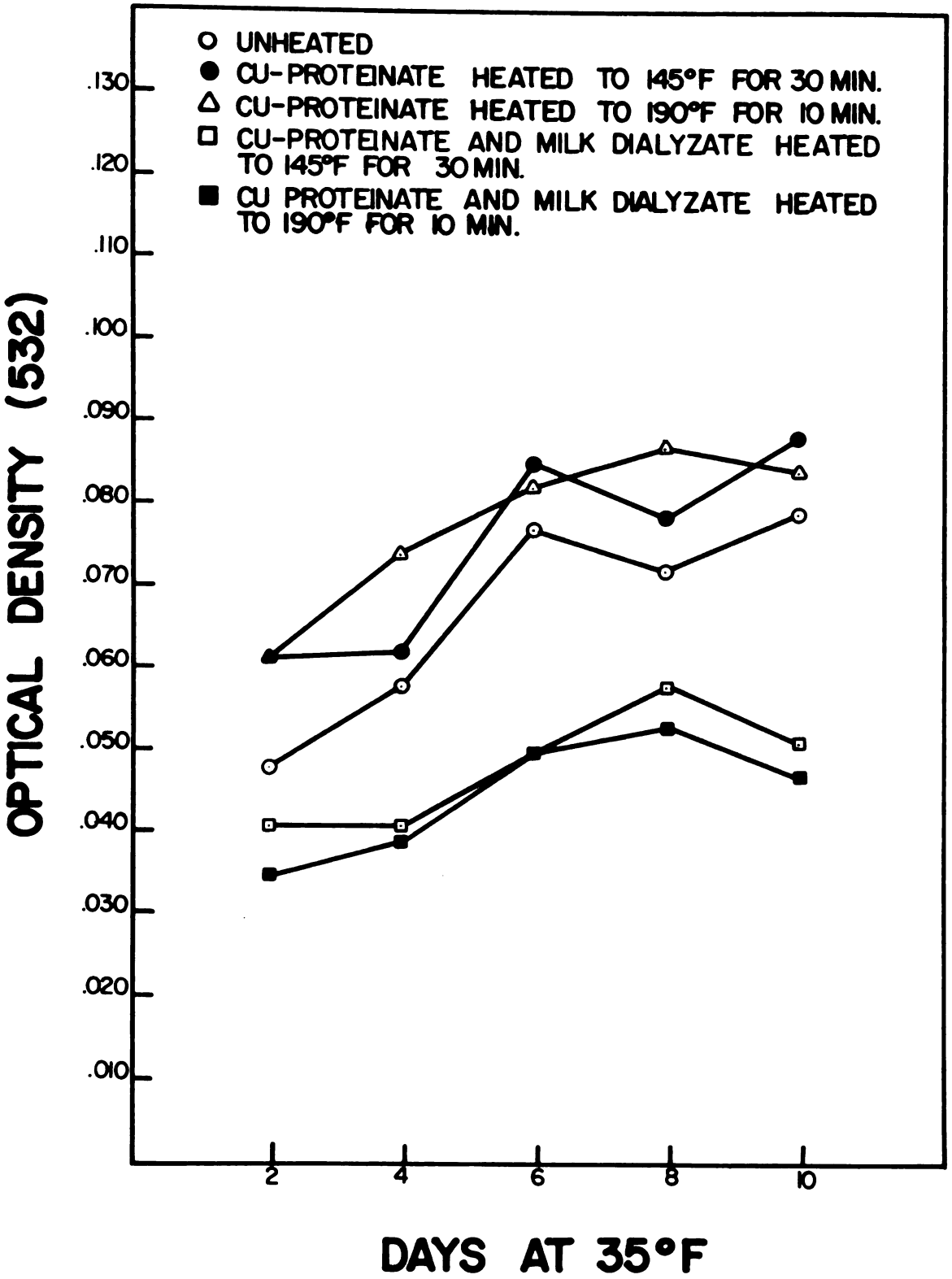

Figure 12a. Lipid oxidation in heated and unheated model systems of copper-fat-globule membrane proteinate, washed fat-globules and milk dialyzate (TBA).

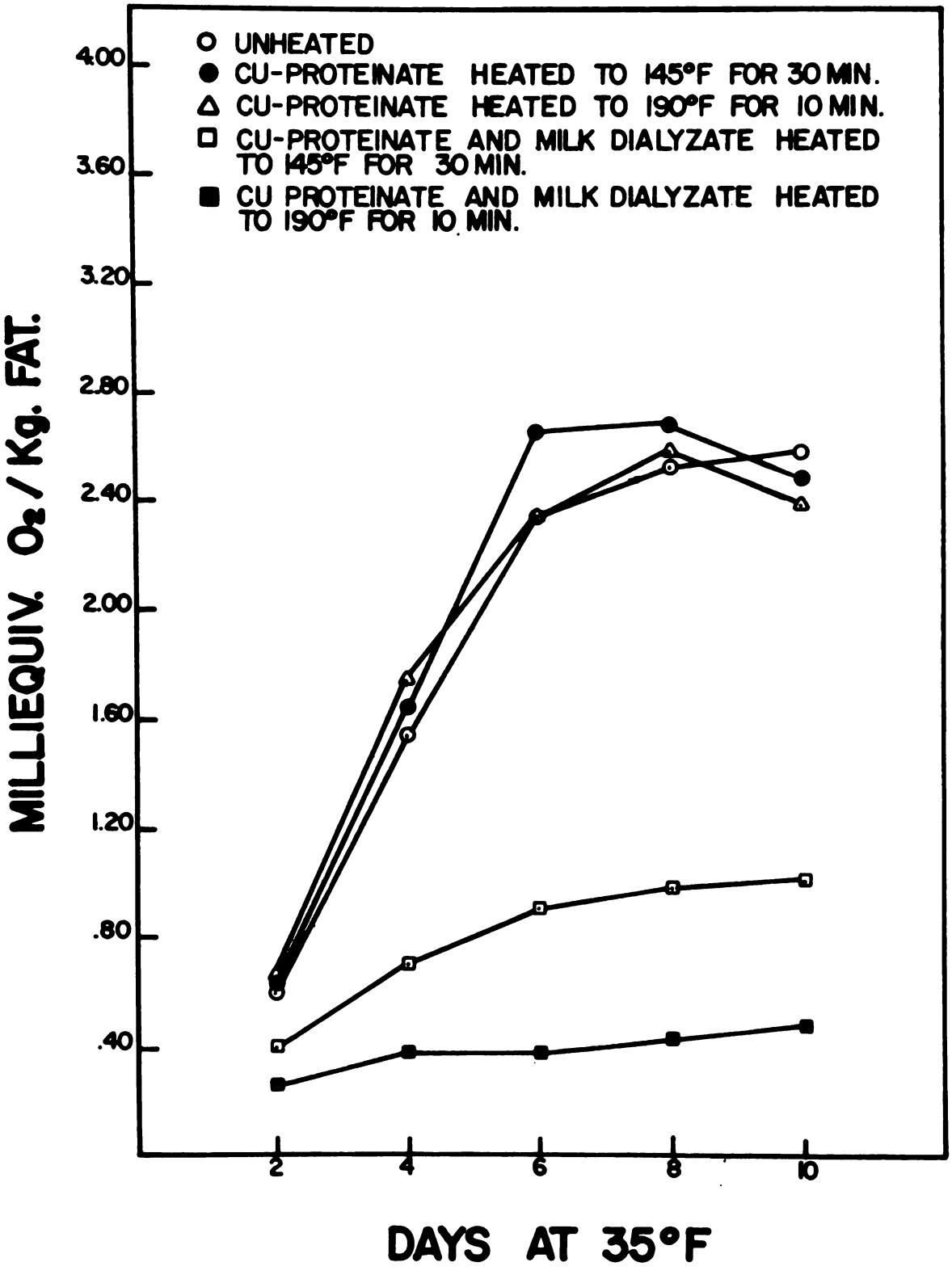

Figure 12b. Lipid oxidation in heated and unheated model systems of copper-fat-globule membrane proteinate, washed fat-globules and milk dialyzate (P.V.).

Unlike the results shown in Figure 11a, the extent of oxidation decreased in both heated systems (copper proteinate, and model system of washed fat-globules and milk dialyzate) after the termination of the storage period (Figure 12a). The results indicated in Figure 12b are essentially similar to those presented in Figure 11b in that peroxide values of systems containing heated metal proteinate and heated fat-globules exhibited minimal oxidation. The results shown here agreed with those presented in Figures 6a, 7a, 8, 9, 10, 11a, 11b and 12a in that the high heat treatments for short time had the maximum inhibiting effect on the extent of oxidation. Although minimal oxidation occurred in systems in which both copper proteinate, and fat-globules and milk dialyzate had been heated, the systems containing heated metal proteinate and unheated fat-globules did not show any decrease in oxidation.

Inspection of the data in Figures 11a and 12a indicates that there is beneficial antioxidant effect of heating the milk dialyzate and fat-globules. Heating the metal proteinate itself without subjecting the fat-globules and milk dialyzate to similar heat treatment did not reduce oxidation in the system. Such evidence would appear to substantiate the data of Sargent (96) and others (77) who found that heating fat-globules in their natural environment caused a shift in trace metal distribution in

the milk. Heating fat-globules apparently causes a significant alteration in either the configuration or concentration of autoxidizable material at the fat-globule interface. The decrease in oxidation in both heated systems (copper proteinate, and washed fat-globules and milk dialyzate) is also attributed to the reduction in the pro-oxidant activity of copper proteinates. This decrease in the pro-oxidant properties of copper-proteinate added to heated model system of washed fat-globules and milk dialyzate as compared to this property when copper proteinate was added to the unheated model system, is probably due to binding of copper by some reducing substances formed during the heat treatment. The individual heating of copper proteinates and model system of washed fat-globules and milk dialyzate did not seem to yield results significantly different than those obtained from the experiments where both systems (copper proteinate, and washed fat-globules and milk dialyzate) were heated together.

SUMMARY

The binding of copper by α-casein, β-casein, β-lactoglobulin, fat-globule membrane protein, and centrifuged micellar casein was studied by equilibrium dialysis at pH 6.5. Solutions of the above proteins, in concentrations of 2.436, 1.205, 1.420, 3.000, and 2.000 mg/l, respectively, were placed in washed dialysis bags and equilibrated for 72 hours against each of four copper solutions. The concentration of copper outside the membrane was 16x, 32x, 64x, or 80 x 10^{-6}M. Plots of moles of copper bound per mole of protein versus the log of the unbound copper yielded typical curves. The results indicated the maximum copper binding capacity of micellar casein and fat-globule membrane protein. The affinity of the proteins to copper at pH 6.5 was decreased in the following order: centrifuged micellar casein, fat-globule membrane protein, sodium α-caseinate, β-lactoglobulin and sodium β-caseinate.

Model systems of copper proteinate of micellar casein, fat-globule membrane protein, sodium α-casein, sodium β-casein and β-lactoglobulin were suspended in a model system of washed fat-globules and milk dialyzate. The model system without copper was designated as the

control. The model systems with copper and without copper were subjected to heat treatments of 145°F for 30 minutes and 190°F for 10 minutes. After cooling, the samples were stored at 35°F for 10 days. At 2-, 4-, 6-, 8- and 10-day intervals oxidation was studied by conducting TBA and peroxide value tests. Data were shown in graphs by plotting the TBA and PV values against the storage time. Results indicated that the rate of oxidation was higher in unheated systems than in those heated to 145°F for 30 minutes. The systems heated to 190°F for 10 minutes exhibited the least amount of oxidation. The extent of oxidation in most of the model systems under investigation was directly proportional to their storage period.

Likewise, in systems in which the protein or metallo-protein were heated separately and then combined with a system of washed fat globules and milk dialyzate which also had been heated separately, lower levels of oxidation, as measured by TBA values, were noted in heated systems. In systems in which the metallo-protein had been heated, but the fat-globules and milk dialyzate had not been heated, such antioxidant properties were not observed.

These results indicate that much of the beneficial effects of heat treatment arise from the effects of heat on materials adsorbed at the fat-globule.

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APPENDIX

APPENDIX

A. Copper-Free Glassware

All glassware used for copper analysis was immersed in concentrated nitric acid for two minutes, followed by five thorough rinsings with double-distilled water. The electrical conductivity of redistilled water was always less than 1.0μ mhos.

Reagents needed for copper analysis and standard copper solution were prepared by employing the method of King and Dunkley (52), followed by Sargent (96).

B. Copper Determination

Preparation of standard curve

1. Ten milliliters of copper solutions of the following concentrations 16×10^{-6} M, 32×10^{-6} M, 64×10^{-6} M, and 80×10^{-6} M were pipetted successively into four 125 ml glass extraction flasks.
2. Ten milliliters of ammonium citrate solution was added into each extraction flask.
3. Two milliliters of 2% aqueous sodium diethyldithiocarbamate solution was added; the flasks were shaken vigorously for 2 minutes, and 15 minutes was allowed for completion of reaction.
4. Ten milliliters of reagent grade carbon tetrachloride was added, the flasks were shaken for 5 minutes and set aside for 10 minutes.

5. About 4 ml of the carbon tetrachloride layer was transferred to a cuvette, and the optical density at 435 mμ was determined with a Beckman Model DU-2 spectrophotometer, slit width 0.04 mm.

This procedure was followed in the various copper-protein binding experiments in the present investigation.

TABLE 1.--Binding of copper by micellar casein.[a]

Moles bound Cu / Moles total protein	Log copper free
143.5	-4.50
100.0	-4.66
57.5	-5.19
25.0	-5.70

[a]Plotted in Figure 1.

TABLE 2.--Binding of copper by fat-globule membrane protein.[a]

Moles bound Cu / Moles total protein	Log copper free
70	-4.34
57.5	-4.49
25	-4.89
15	-5.46

[a]Plotted in Figure 2.

TABLE 3.--Binding of copper by sodium α-caseinate.[a]

Moles bound Cu / Moles total protein	Log copper free
13.1	-4.53
11	-4.71
7	-5.46
3.5	-5.70

[a]Plotted in Figure 3.

TABLE 4.--Binding of copper by sodium β-caseinate.[a]

Moles bound Cu / Moles total protein	Log copper free
3.95	-4.46
3.30	-4.56
2.50	-5.15
1.00	-5.7

[a]Plotted in Figure 4.

TABLE 5.--Binding of copper by β-lactoglobulin.[a]

Moles bound Cu / Moles total protein	Log copper free
4.63	-4.41
4.00	-4.54
3.00	-5.10
1.40	-5.52

[a]Plotted in Figure 5.

TABLE 6.--The TBA values obtained from lipid in model systems of copper-micellar caseinate, washed fat-globules and milk dialyzate.[a]

Sample Treatment	Days at 35°F				
	2	4	6	8	10
Control unheated	0.056	0.063	0.075	0.068	0.069
Control at 145°F for 30 min.	0.029	0.035	0.044	0.039	0.043
Control at 190°F for 10 min.	0.015	0.018	0.019	0.019	0.019
Cu-P unheated	0.075	0.138	0.254	0.233	0.311
Cu-P at 145°F for 30 min.	0.050	0.074	0.105	0.092	0.167
Cu-P at 190°F for 10 min.	0.048	0.053	0.082	0.080	0.114

[a]Plotted in Figure 6a. Cu-P = copper proteinate.

TABLE 7.--The peroxide values obtained from lipid in model systems of copper-micellar caseinate, washed fat-globules and milk dialyzate.[a]

Sample Treatment	Days at 35°F				
	2	4	6	8	10
Control unheated	1.0665	1.149	1.360	0.328	0.844
Control at 145°F for 30 min.	0.659	0.938	0.938	0.609	1.406
Control at 190°F for 10 min.	0.414	0.436	0.457	0.441	0.661
Cu-P unheated	3.706	9.189	9.024	4.805	3.633
Cu-P at 145°F for 30 min.	1.195	3.141	5.016	7.032	11.300
Cu-P at 190°F for 10 min.	0.891	1.781	2.554	3.422	7.688

[a]Plotted in Figure 6b. Cu-P = copper proteinate.

TABLE 8.--The TBA values obtained from lipid in model systems of copper-fat-globule membrane proteinate, washed fat-globules and milk dialyzate.[a]

Sample Treatment	Days at 35°F				
	2	4	6	8	10
Control unheated	0.023	0.025	0.034	0.033	0.036
Control at 145°F for 30 min.	0.017	0.018	0.029	0.022	0.023
Control at 190°F for 10 min.	0.015	0.016	0.027	0.021	0.022
Cu-P unheated	0.060	0.079	0.108	0.109	0.115
Cu-P at 145°F for 30 min.	0.030	0.047	0.063	0.062	0.070
Cu-P at 190°F for 10 min.	0.025	0.042	0.046	0.045	0.055

[a]Plotted in Figure 7a. Cu-P = copper proteinate.

TABLE 9.--The peroxide values obtained from lipid in model systems of copper-fat-globule membrane proteinate, washed fat-globules and milk dialyzate.[a]

Sample Treatment	Days at 35°F				
	2	4	6	8	10
Control unheated	0.621	0.663	0.488	0.518	0.492
Control at 145°F for 30 min.	0.429	0.406	0.424	0.490	0.433
Control at 190°F for 10 min.	0.424	0.377	0.377	0.401	0.408
Cu-P unheated	1.711	3.770	3.586	2.414	2.719
Cu-P at 145°F for 30 min.	0.774	1.524	1.992	2.086	2.297
Cu-P at 190°F for 10 min.	0.492	0.820	1.055	1.946	1.922

[a]Plotted in Figure 7b. Cu-P = copper proteinate.

TABLE 10.--The TBA values obtained from lipid in model systems of copper sodium-α-caseinate, washed fat-globules and milk dialyzate.[a]

Sample Treatment	Days at 35°F				
	2	4	6	8	10
Control unheated	0.036	0.033	0.046	0.049	0.044
Control at 145°F for 30 min.	0.020	0.025	0.038	0.039	0.036
Control at 190°F for 10 min.	0.018	0.023	0.040	0.037	0.034
Cu-P unheated	0.040	0.060	0.061	0.077	0.074
Cu-P at 145°F for 30 min.	0.039	0.038	0.039	0.056	0.062
Cu-P at 190°F for 10 min.	0.038	0.039	0.041	0.044	0.048

[a]Plotted in Figure 8. Cu-P = copper proteinate.

TABLE 11.--The TBA values obtained from lipid in model systems of copper sodium-β-caseinate, washed fat-globules and milk dialyzate.[a]

Sample Treatment	Days at 35°F				
	2	4	6	8	10
Control unheated	0.026	0.033	0.036	0.040	0.043
Control at 145°F for 30 min.	0.017	0.020	0.024	0.024	0.029
Control at 190°F for 10 min.	0.018	0.020	0.022	0.025	0.028
Cu-P unheated	0.040	0.058	0.085	0.095	0.107
Cu-P at 145°F for 30 min.	0.029	0.035	0.044	0.044	0.054
Cu-P at 190°F for 10 min.	0.028	0.036	0.043	0.042	0.046

[a]Plotted in Figure 9. Cu-P = copper proteinate.

TABLE 12.--The TBA values obtained from lipid in model systems of copper β-lactoglobulin, washed fat-globules and milk dialyzate.[a]

Sample Treatment	Days at 35°F				
	2	4	6	8	10
Control unheated	0.020	0.025	0.025	0.022	0.028
Control at 145°F for 30 min.	0.014	0.021	0.020	0.020	0.025
Control at 190°F for 10 min.	0.013	0.020	0.020	0.019	0.026
Cu-P unheated	0.044	0.062	0.082	0.076	0.080
Cu-P at 145°F for 30 min.	0.029	0.035	0.040	0.038	0.049
Cu-P at 190°F for 10 min.	0.025	0.029	0.035	0.030	0.041

[a]Plotted in Figure 10. Cu-P = copper proteinate.

TABLE 13.--The TBA values obtained from lipid in heated and unheated model systems of copper β-lactoglobulin, washed fat-globules and milk dialyzate.[a]

Sample Treatment	Days at 35°F				
	2	4	6	8	10
Unheated systems	0.043	0.042	0.045	0.056	0.073
Cu-P at 145°F for 30 min.	0.047	0.050	0.041	0.057	0.075
Cu-P at 190°F for 10 min.	0.052	0.062	0.049	0.061	0.075
Cu-P and milk dialyzate at 145°F for 30 min.	0.036	0.034	0.037	0.047	0.065
Cu-P and milk dialyzate at 190°F for 10 min.	0.039	0.032	0.028	0.042	0.058

[a]Plotted in Figure 11a. Cu-P = copper proteinate.

TABLE 14.--The peroxide values obtained from lipid in heated and unheated model systems of copper β-lactoglobulin, washed fat-globules and milk dialyzate.[a]

Sample Treatment	Days at 35°F				
	2	4	6	8	10
Unheated systems	0.567	0.727	1.031	1.125	1.641
Cu-P at 145°F for 30 min.	0.497	0.727	0.844	1.078	1.360
Cu-P at 190°F for 10 min.	0.483	0.717	0.797	0.938	1.266
Cu-P and milk dialyzate at 145°F for 30 min.	0.548	0.624	0.891	0.938	1.172
Cu-P and milk dialyzate at 190°F for 10 min.	0.441	0.605	0.516	0.563	0.563

[a]Plotted in Figure 11 b. Cu-P = copper proteinate.

TABLE 15.--The TBA values obtained from lipid in heated and unheated model systems of copper-fat globule membrane proteinate, washed fat-globules and milk dialyzate.[a]

Sample Treatment	Days at 35°F				
	2	4	6	8	10
Unheated systems	0.048	0.058	0.077	0.072	0.079
Cu-P at 145°F for 30 min.	0.061	0.062	0.085	0.078	0.088
Cu-P at 190°F for 10 min.	0.061	0.074	0.082	0.087	0.084
Cu-P and milk dialyzate at 145°F for 30 min.	0.035	0.039	0.050	0.053	0.047
Cu-P and milk dialyzate at 190°F for 10 min.	0.046	0.041	0.050	0.058	0.051

[a]Plotted in Figure 12a. Cu-P = copper proteinate.

TABLE 16.--The peroxide values obtained from lipid in heated and unheated model systems of copper-fat-globule membrane proteinate, washed fat-globules and milk dialyzate.[a]

Sample Treatment	Days at 35°F				
	2	4	6	8	10
Unheated systems	0.609	1.547	2.344	2.532	2.578
Cu-P at 145°F for 30 min.	0.637	1.641	2.625	2.672	2.485
Cu-P at 190°F for 10 min.	0.646	1.735	2.344	2.578	2.391
Cu-P and milk dialyzate at 145°F for 30 min.	0.403	0.703	0.891	0.984	1.031
Cu-P and milk dialyzate at 190°F for 10 min.	0.267	0.375	0.375	0.422	0.469

[a]Plotted in Figure 12b. Cu-P = copper proteinate.