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To the Graduate Council:

I am submitting herewith a dissertation written by Sharon Lynn Melton entitled "Protein-Phospholipid Interactions in Heated Phospholipid Emulsions Containing Bovine Myoglobin or Sarcoplasmic Proteins." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science.

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Major Professor

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Graduate Studies and Research

PROTEIN-PHOSPHOLIPID INTERACTIONS IN HEATED PHOSPHOLIPID EMULSIONS CONTAINING BOVINE MYOGLOBIN OR SARCOPLASMIC PROTEINS

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Sharon Lynn Melton

December 1973

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ABSTRACT

A simplified procedure for determination of myoglobin concentration in aqueous muscle extract was developed. The procedure was used in an investigation of possible interactions between phospholipids and bovine myoglobin, either isolated or as a component of sarcoplasmic proteins. Investigation of the possible interactions was carried out as follows. Solutions containing myoglobin or sarcoplasmic proteins and emulsions (phospholipid, sarcoplasmic protein-phospholipid, and myoglobin-phospholipid) were heated from 25°C to end point temperatures up to 77°C. The following measurements were made on all protein dispersions: (1) pH; (2) percent protein remaining dispersed; (3) myoglobin concentration; (4) available lysine, basic group and acidic group equivalents of dispersed and total protein; and (5) densitometric tracings of electrophoretic patterns of dispersed proteins. The following measurements were made on protein-containing emulsions: (1) quantities of free and bound lipid associated with dispersed, precipitated and total protein; (2) quantities of phospholipid classes (lysolecithin, sphingomyelin, lecithin, cephalin, ninhydrin-positive products) in the free and bound lipid; and (3) decomposition of phospholipids to water soluble phosphorus compounds. The following measurements were conducted only on phospholipid emulsions: (1) lipid concentration; (2) phospholipid classes; and (3) decomposition of phospholipids.

Interactions between phospholipids and myoglobin, either isolated or as a component of sarcoplasmic proteins, and between phospholipids and total sarcoplasmic proteins were found. The interactions between phospholipids and proteins in the sarcoplasmic extract involved mainly weak polar bonds but interaction between phospholipids and isolated myoglobin involved both polar and possibly non-polar bonds as well as either covalent or strong complex bonds between phospholipids and the basic groups, especially the ϵ-NH_2 of lysine in the myoglobin. These interactions formed phospholipid-protein complexes, altered the amount of protein precipitated by heat and caused the protein after heating to be less stable to a higher pH (7.00) than if heated alone.

Densitometric tracings of the electrophoretic patterns of sarcoplasmic proteins showed the disappearance of certain proteins as a function of increasing end point temperature and showed differences in myoglobin concentrations in heated solutions and emulsions. Densitometric tracings of the electrophoretic pattern of myoglobin in dispersions with and without phospholipids indicated formation of a myoglobin-phospholipid complex that would not migrate in a gel at pH 8.1 until it was heated.

Heating the emulsions caused approximately 1 percent of the total phospholipids to decompose to water soluble phosphorus compounds in phospholipid emulsions and approximately 10 percent of the total phospholipids in sarcoplasmic protein emulsions to change to ninhydrin-positive products tentatively identified as lysocephalin and a geometric

isomer of cephalin. These quantities, respectively, were calculated from significant ($P < 0.05$) increases in water soluble phosphorus and in ninhydrin-positive products.

Heating the sarcoplasmic protein-phospholipid emulsions generally caused the following significant changes ($P < 0.05$): (1) a decrease in free lipid (and a decrease in quantities of lecithin and cephalin in that free lipid) associated with dispersed protein and (2) a decrease in the quantity of lecithin and an increase in the quantities of lysolecithin and ninhydrin-positive products in lipid bound to dispersed protein. Heating the myoglobin-phospholipid emulsions significantly ($P < 0.05$) affected the amount of lecithin in lipid bound to dispersed protein. Heating from 25 to 65°C caused an increase and from 65 to 77°C, a decrease; the quantity at 77°C was significantly smaller than the quantity at 25°C. Heating the emulsions from 25°C to the first end point temperature caused the formation of a precipitate containing protein associated with free and bound lipid in which all phospholipid classes were represented.

When the protein of heated emulsions was separated into dispersed and precipitated protein and the bound and free lipids associated with dispersed or precipitated protein were separated into phospholipid classes, a partitioning of the phospholipids was found. Phospholipid classes were distributed differently between bound and free lipid associated with dispersed or precipitated protein. The partitioning occurred whether the emulsions contained total sarcoplasmic protein or myoglobin.

The color of cooked meat depends largely upon the nature and amount of myoglobin derivatives present. Therefore, interactions between phospholipids and myoglobin, such as those observed in this study, could affect directly the color of cooked meat.

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CHAPTER I

GENERAL INTRODUCTION

Today the color of cooked meat is still used as an indicator of the degree of cookery to which meat has been subjected. Draudt (1969) points out that color is likely to be a rather poor indicator of the actual internal temperature that meat attains during cooking because of wide differences in the heat precipitation of various fresh meat pigments.

The final color of cooked lean meat depends largely upon the nature and amount of myoglobin derivatives and decomposition products that are present. The changes in the nature and amount of myoglobin derivatives present in cooked meat are determined by the type, rate and extent of cooking. Cooked meats that have been heated to internal temperatures of 50-70°C have decreasing pink color with increasing internal temperature. The pink color is attributable to oxymyoglobin which is water-extractable (Bernofsky et al., 1959). Meats cooked to an internal temperature of 75°C or above are brown or gray in color because of a number of pigments including denatured heme compounds which are not water-extractable.

It has been established that myoglobin derivatives precipitate in meat and water extracts of muscle (at temperatures of 60°C and above) about 15-30°C below the temperature required in pure solution at the same pH (Draudt, 1969; Ledward, 1971). It has been reported that the myoglobin precipitation at these relatively low temperatures probably

results from myoglobin interactions with other proteins denaturing at 60-65°C (Ledward, 1971).

Phospholipids, which make up about 10-30 percent of the total lipid of muscle, are affected by heating. Turkki and Campbell (1967) reported that drip phospholipids from cooked beef consisted largely of cephalin and the cooked meat contained significantly less cephalin than the raw meat. Terrell et al. (1968) reported that broiling affected the fatty acids of phospholipids in bovine muscle. Other investigators reported that heating raw milk decreased phospholipid phosphorus content (Nakanishi and Kaya, 1970a,b), and Macy and coworkers (1964) concluded that hydrolysis of phospholipids occurred during the heating of meat.

Interactions between lipids and proteins have been studied extensively in flour dough systems and in biological systems (Fullington, 1969). Phospholipids also have been shown to bind with soybean protein (Rzhekhin and Krasilnov, 1965) and to interact with fish actomyosin (Ikeda and Taguchi, 1968).

Reports were not found of studies in which interactions between myoglobin and nonprotein muscle constituents such as lipids were investigated. It is possible that interactions between myoglobin and phospholipids occur during the cooking of meat and that these interactions could protect or destabilize myoglobin to heat precipitation.

Investigators who have determined myoglobin concentration in bovine muscle or in cooked beef have extracted the myoglobin with water and then determined myoglobin concentration in the water extract. Procedures used to determine myoglobin concentration in water extracts vary.

Some investigators have measured the total hemoprotein (myoglobin and hemoglobin) in the water extract and neglected the hemoglobin concentration (Ledward, 1971). However, hemoglobin concentration in bovine muscle was reported as 20-30 percent of the total hemoprotein (Paul, 1972a). Other investigators have separated the myoglobin from hemoglobin in 3 M phosphate solution and then measured the myoglobin concentration. Some of the investigators who separated myoglobin from hemoglobin added a saturated basic lead acetate to the water extract prior to making it 3 M in phosphate (Ginger et al., 1954; Rickansrud and Henrickson, 1967) but others did not add saturated basic lead acetate (Gőtze, 1969). Most of the investigators also prepared cyanoderivatives of the hemoproteins and then calculated the concentration from the absorbance of the cyanoderivatives at 540 nm. The reported isosbestic point, 525 nm, for all forms of myoglobin and hemoglobin was used to determine hemoprotein concentration in water extracts of bovine muscle (Ledward, 1971).

In an investigation of myoglobin in heated water extracts of bovine muscle it would be advantageous to have a simplified procedure for the accurate determination of myoglobin concentration. However, different steps in the reported procedures of determining myoglobin concentration should be investigated more fully so that the steps which add to the precision of the measurement could be incorporated into any simplified procedure.

Polyacrylamide disc gel electrophoresis was used in the study of bovine sarcoplasmic (water-soluble) proteins (Awad et al., 1968;

Laakkonen et al., 1970). Recently, investigators who have studied milk proteins electrophoretically have used densitometric tracings of disc gel electrophoretograms to estimate quantitatively the concentrations of individual milk proteins (Bechtle and Claydon, 1971; Dill et al., 1971). This suggests the possibility of using a densitometric tracing of an electrophoretic pattern of sarcoplasmic proteins extracted from bovine muscle to estimate quantitatively myoglobin concentration.

Additional knowledge of the interaction of myoglobin with other muscle constituents is needed in order to understand more clearly the contribution of the denatured hemoproteins to the color of meat that is cooked to varying end point temperatures. Previously cited evidence concerning the reactivity of myoglobin and of phospholipids suggests the advisability of investigating possible interactions between myoglobin and phospholipids.

Before an investigation of possible interactions could be attempted it was necessary to find an accurate simplified procedure for the determination of myoglobin concentration in water-extracts of bovine muscle. In a search for this procedure the following were investigated: (1) reported procedures, (2) a proposed simplified procedure based on the reported procedures and (3) the possibility of using polyacrylamide disc gel electrophoresis as a quantitative tool to determine myoglobin concentration.

Investigation of possible interactions between phospholipids and myoglobin, either isolated or as a component of sarcoplasmic proteins,

(water extract of bovine muscle) and between sarcoplasmic proteins and phospholipids was conducted as follows. Aqueous dispersions of bovine myoglobin, myoglobin-phospholipid emulsions, sarcoplasmic proteins (water extracts) and sarcoplasmic protein-phospholipid emulsions were heated under a nitrogen atmosphere from 25°C to various end point temperatures up to 77°C. The following measurements were made on each dispersion at 25°C and at each end point temperature: (1) percent protein remaining dispersed at the pH of the dispersion and after pH of the dispersion that had been heated was adjusted to 7.00; (2) myoglobin concentration by isolation in 3 M phosphate solution; (3) pH; (4) available lysine, basic and acidic groups of dispersed protein and of total protein in the mixture; and (5) polyacrylamide disc gel electrophoretic pattern of dispersed proteins. The following measurements also were made on the emulsions at 25°C and at each end point temperature: (1) quantities of free and bound lipid associated with dispersed, precipitated and total protein in the emulsion; (2) quantities of the phospholipid classes (lysolecithin, sphingomyelin, lecithin, cephalin and other ninhydrin-positive products) in the free and bound lipid associated with dispersed, precipitated and total protein in the emulsions; (3) increase in water soluble phosphorus as an indicator of phospholipid decomposition to water soluble phosphorus compounds; and (4) lipid recovery in the emulsions.

In addition, decomposition of phospholipids in the absence of protein was investigated by heating phospholipid emulsions under a nitrogen atmosphere to the same end point temperatures to which the

protein-lipid emulsions were heated. The following measurements were made on the phospholipid emulsions: (1) lipid concentration; (2) phospholipid classes; and (3) increase in water soluble phosphorus.

The results of these investigations are reported in three separate chapters in this dissertation. Each chapter contains the following subdivisions: (1) an introduction; (2) materials and methods; (3) results and discussion; and (4) summary. A general summary of all results is reported in Chapter V.

CHAPTER II

DETERMINATION OF MYOGLOBIN CONCENTRATION

Introduction

Quantitative determination of myoglobin in aqueous muscle extracts has been of interest since the 1930s. Current procedures for the determination of myoglobin in muscle extracts were reported by Rickansrud and Henrickson (1967) and Gőtze (1969). The procedure of Rickansrud and Henrickson (1967) was a modification of the methods of Drabkin (1950) and Ginger et al. (1954). This procedure involved: (1) precipitation of foreign proteins with a saturated basic lead acetate solution (Ginger et al., 1954); (2) separation of myoglobin from hemoglobin and other proteins in a 3 M phosphate solution, pH 6.6 (Green, 1931; Morgan, 1935); (3) conversion of the isolated myoglobin to the cyanometmyoglobin derivative (Drabkin and Austin, 1936); and (4) spectrophotometric determination of the cyano-derivative concentration at 540 nm (molar absorptivity, 11.3 mM/l, when cell path length = 1.00 cm, Drabkin, 1950). This method is long and laborious especially if several measurements of myoglobin in water extracts of muscle are made. Basic lead acetate was not used in the procedure reported by Gőtze (1969). His procedure involved: (1) separation of myoglobin from hemoglobin in a 3 M phosphate buffer; (2) conversion of the myoglobin to the cyano-derivative; and (3) spectrophotometric determination of the concentration of the cyano-derivative.

Conversion of the myoglobin to the cyano-derivative in both procedures requires addition of $K_3Fe(CN)_6$ and KCN to the myoglobin-containing solution (Drabkin and Austin, 1936) and may be an unnecessary step. Ledward (1971) measured the myoglobin concentration in myoglobin solutions at a wave length of 525 nm. This wavelength was found to be an isosbestic point for the oxy-, reduced and oxidized forms of myoglobin (molar absorptivity, 7.5 mM/l when cell path length = 1.00; Bowen, 1949).

In recent years there have been reports of studies in which polyacrylamide disc gel electrophoretograms of bovine sarcoplasmic proteins were illustrated (Awad et al., 1968; Laakkonen et al., 1970). Densitometric tracings of polyacrylamide disc gel electrophoretograms were used to estimate quantitatively the concentrations of individual components in skim milk proteins and in whey proteins (Dill et al., 1971; Bechtle and Claydon, 1971). Therefore, it is possible that polyacrylamide disc gel electrophoretograms of sarcoplasmic proteins extracted from bovine muscle might be used to estimate the myoglobin concentration in the extracts.

The Rickansrud and Henrickson (1967) procedure was used in this laboratory in a preliminary investigation of myoglobin concentration in water extracts of bovine muscle. When their procedure was followed, the use of two different saturated basic lead acetate solutions resulted in two widely different values for myoglobin concentration in the same water extract of bovine muscle. The saturated solutions were prepared from basic lead acetate from two different sources (Mallinckrodt and

Fisher Scientific Co.) similar in composition according to the labels. A wide difference was found between the specific gravities of the two saturated basic lead acetate solutions although both solutions were "saturated" in the sense that an equilibrium appeared to be established between solid component and solution. Nemedy (1935) reported that the specific gravity of basic lead acetate solutions was directly proportional to the lead content in solution.

Investigators who used "saturated" basic lead acetate solutions in their procedure for determination of myoglobin concentration (Rickansrud and Henrickson, 1967; Ginger et al., 1954) did not report the source or composition of the basic lead acetate or the procedure for making the "saturated" solution. The only basic lead acetate that was reported to have limited solubility in water was lead acetate, dibasic, with a chemical formula of $Pb(C_2H_3O_2)_2 \cdot 2Pb(OH)_2$. This compound is used as a clearing agent in sugar analysis and its solubility was reported as 5.55 g/100 ml cold water (LIA, 1952). The labels of the basic lead acetate used in the preliminary investigation did not give a chemical formula, but the label on the Mallinckrodt basic lead acetate stated that it was for sugar analysis. The labels of both sources reported the basic lead content of the compound expressed as PbO. This content was 33 percent for the compound from Mallinckrodt and 32.7 percent for the compound from Fisher Scientific Co.

It was assumed that each of the basic lead acetate compounds in the preliminary investigation was the lead compound of limited solubility in water. When the saturated solutions of these compounds were

prepared, amounts of the basic lead acetate added per 100 ml of cold water were much greater than the reported solubility of lead acetate, dibasic. Unfortunately records were not obtained of the exact quantity of either compound added per 100 ml of water.

It was observed also during the preliminary investigation that it was difficult to dissolve the large amount of dry phosphate salts added to the myoglobin-containing solutions to make the solutions 3 M in phosphate (Rickansrud and Henrickson, 1967). It was necessary to stir the myoglobin-containing solutions vigorously for several minutes to make certain all of the added salts were dissolved.

Because of what seemed to be unnecessary steps in the procedures of Rickansrud and Henrickson (1967) and Götze (1969) and the observations of the preliminary investigation, it was decided that a simplified procedure for the determination of myoglobin concentration might be possible. This procedure would include: (1) elimination of the addition of saturated basic lead acetate solutions (already eliminated by Götze, 1969); (2) addition of a concentrated phosphate solution instead of dry phosphate salts to make the myoglobin-containing solutions 3 M in phosphate; (3) elimination of the preparation of the cyano-derivative; and (4) spectrophotometric estimation of the myoglobin concentration in the extracts by absorbance at 525 nm (Bowen, 1949).

This study was initiated: (1) to investigate the effect of "saturated" basic lead acetate solutions on determination of myoglobin concentration by the procedure of Rickansrud and Henrickson (1967); (2) to compare myoglobin concentration as determined from absorbance

at the isosbestic point, 525 nm, with values obtained from absorbance at 540 nm, the wavelength of cyanometmyoglobin absorbance; (3) to determine the feasibility of a simplified procedure for the estimation of myoglobin concentration in water extracts of bovine muscle; and (4) to determine whether a densitometric tracing of the polyacrylamide disc gel electrophoretic pattern of water-soluble proteins extracted from bovine muscle can be used to determine quantitatively the myoglobin concentration in bovine water-soluble proteins.

Materials

Two sources of basic lead acetate were used: Mallinckrodt basic lead acetate, analytical reagent, for sugar analysis, basic lead content expressed as PbO, 33 percent, and Fisher Scientific basic lead acetate, reagent grade, basic lead content expressed as PbO, 32.7 percent.

Horse-heart myoglobin was purchased from Nutritional Biochemicals.

A Bausch and Lomb Spectronic 20 colorimeter, a direct-reading grating spectrophotometer, with a circular cell of 1.17 cm light path length was used for spectrophotometric analysis.

A Lourdes Model A-2 Beta-fuge, refrigerated centrifuge, with a 9RA head was used for all centrifugation.

Purified bovine myoglobin. Purified bovine myoglobin was prepared from a sarcoplasmic protein extract of bovine ground round by a modification of the Quinn et al. (1964) method. Sarcoplasmic proteins

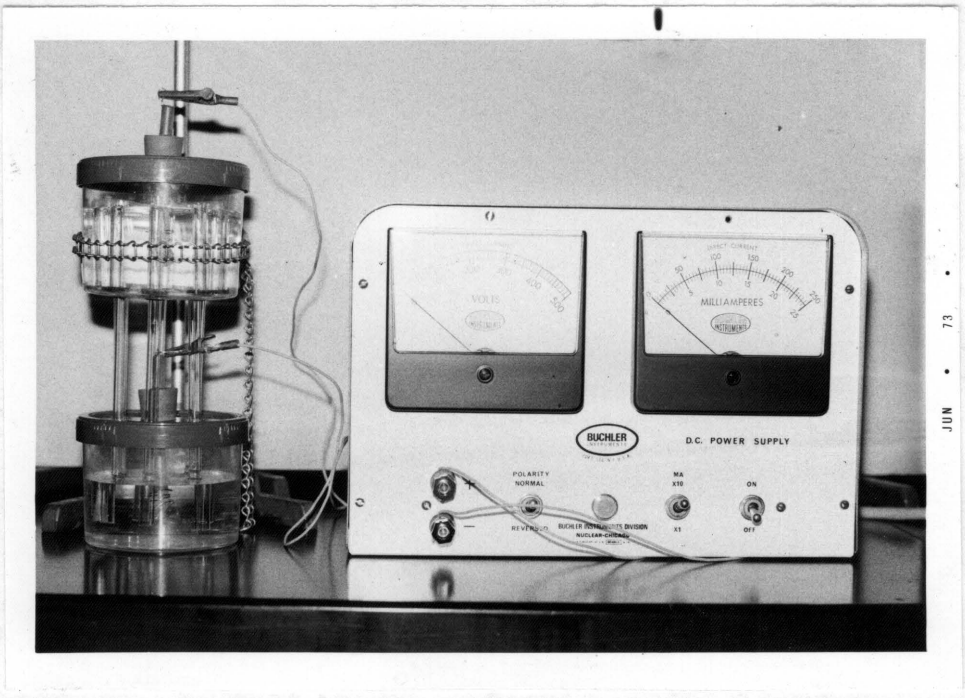
(water-soluble) were extracted by blending 150 ml of cold (1°C) distilled water with 100 g of bovine ground round for 4 minutes in a Waring blendor at medium speed. The meat slurry was centrifuged at 1°C for 10 minutes at 12,800 x G and the supernatant decanted through cheese cloth to remove solidified fat. When 2-4 liters of this extract had been prepared solid ammonium sulfate was added to 65 percent saturation. The precipitate formed was removed by centrifugation at 41,300 x G for 15 minutes at 1°C and discarded. The supernatant then was saturated completely with ammonium sulfate and the myoglobin-containing precipitate was collected by centrifugation at 41,300 x G for 15 minutes at 1°C. The precipitate was dissolved in a small amount of cold distilled water and again saturated with ammonium sulfate. The myoglobin-containing precipitate was collected by centrifugation as above and redissolved in cold distilled water. The precipitation of myoglobin in saturated ammonium sulfate was repeated for a third time, and the myoglobin precipitate was again collected by centrifugation and dissolved in cold distilled water. The myoglobin-containing solution was dialyzed against cold distilled water (with stirring) until it was free from sulfate. It was judged to be free from sulfate when no noticeable white haze (precipitated $BaSO_4$) could be detected in a sample of the dialysate to which $BaCl_2$ was added. The myoglobin-containing solution also was judged free of ammonium sulfate by non-protein nitrogen analysis. The myoglobin (in the form of metmyoglobin) was identified by its spectrum and examined for purity by disc gel electrophoresis. The solutions containing the purified myoglobin were stored at -20°C.

Bovine sarcoplasmic proteins. Bovine sarcoplasmic proteins were extracted by the procedure described for purified myoglobin preparation with elimination of ammonium sulfate fractionation. After the cold water extract was decanted through cheese cloth, it was filtered through Whatman No. 1 filter paper with suction. The filtrate was dialyzed against cold distilled water with stirring at 1°C for 24 hours to remove salts and non-protein nitrogen compounds. A precipitate that formed during dialysis was separated by centrifugation and discarded. The electrophoretic pattern of the water-soluble proteins was determined within 36 hours of extraction.

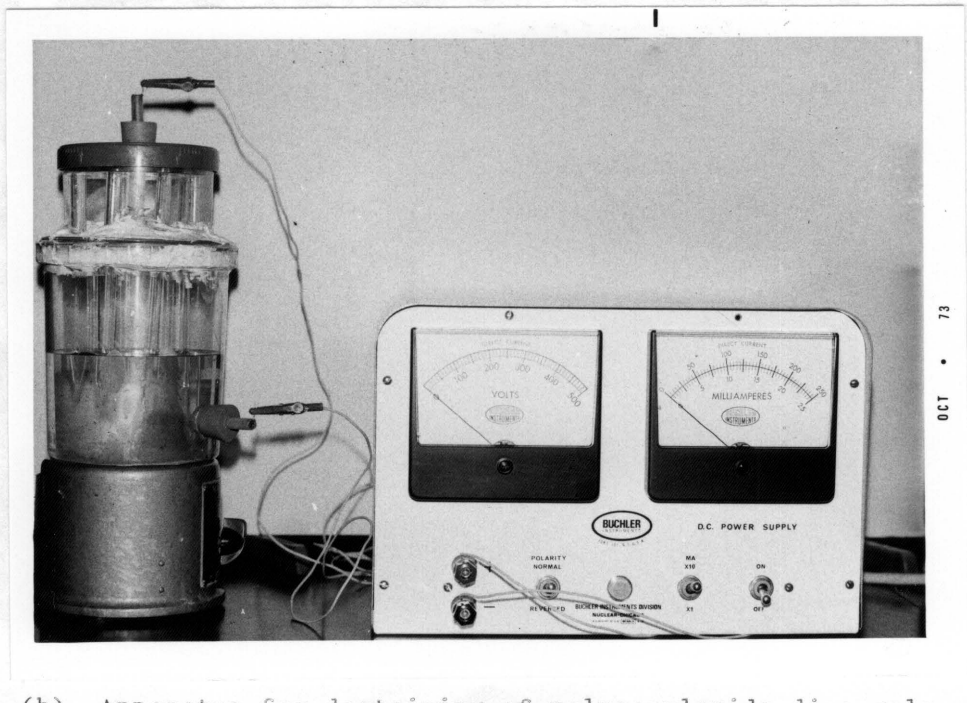
Methods

Polyacrylamide disc gel electrophoresis. Polyacrylamide disc gel electrophoresis of the sarcoplasmic proteins was carried out in an apparatus (Figure 1) constructed in this laboratory according to Nerenberg (1966). A modification of the method described by Nerenberg for the separation of serum proteins was used to separate the bovine sarcoplasmic proteins. The modifications were a 7.5 percent polyacrylamide gel 0.6 cm by 15 cm long instead of a 6 percent polyacrylamide gel 0.6 cm by 12.5 cm long and a glycine-tris buffer of pH 8.1 instead of pH 7.9 for the stock chamber buffer. Nerenberg's (1966) procedure was a simplified version of the Davis (1964) procedure.

During the electrophoretic run (1 hour and 50 minutes) the current per tube dropped from 10 to 2 mA. At the end of the run, the gels were removed from the glass tubes; the position of the myoglobin bands



(a) Apparatus for polyacrylamide disc gel electrophoresis



(b) Apparatus for destaining of polyacrylamide disc gels.

Figure 1. Apparatus constructed for polyacrylamide disc gel electrophoresis and destaining of gels according to the instructions of Nerenberg (1966).

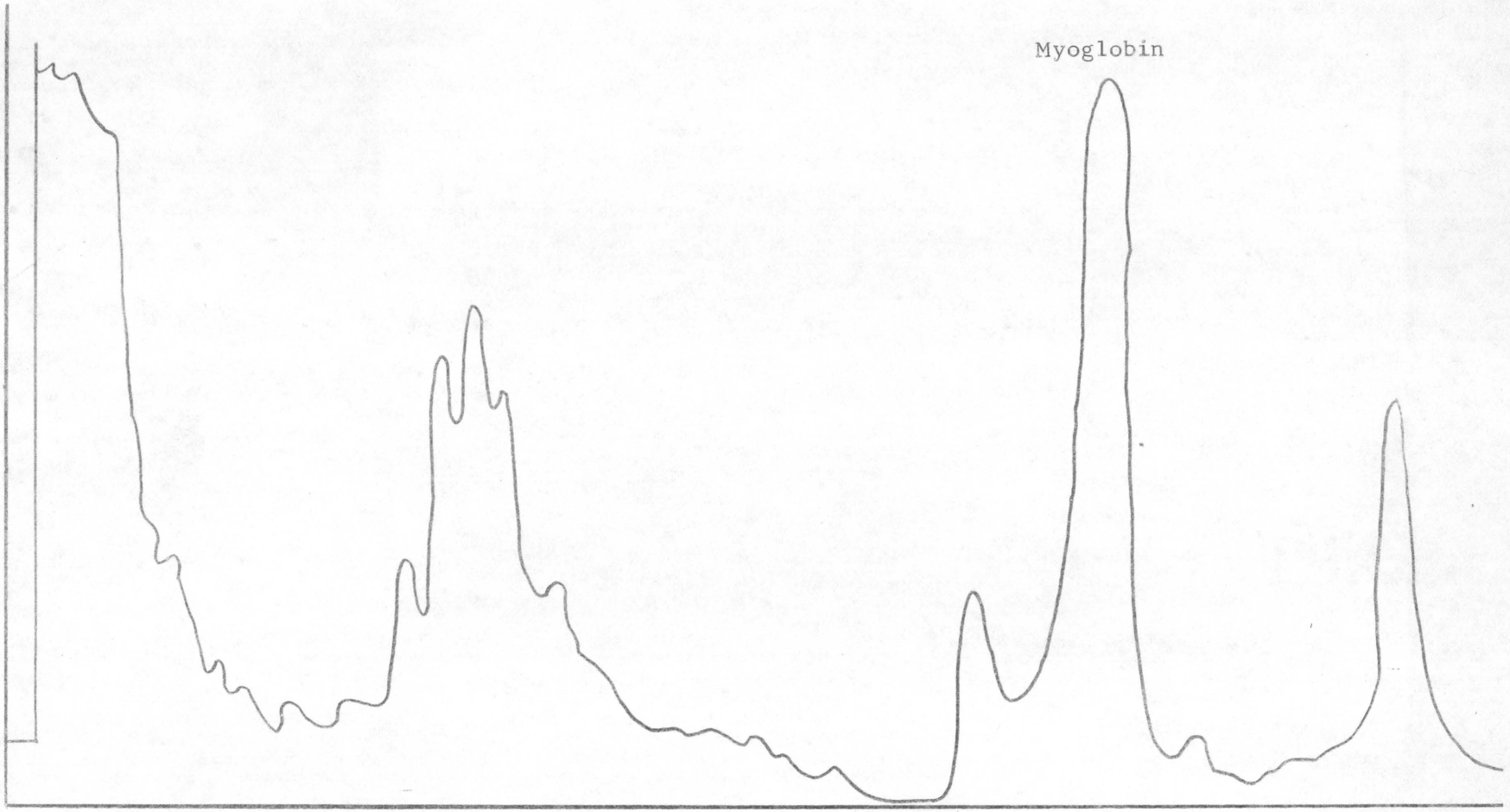


Figure 2. Densitometric tracing of electrophoretic pattern of bovine sarcoplasmic proteins.

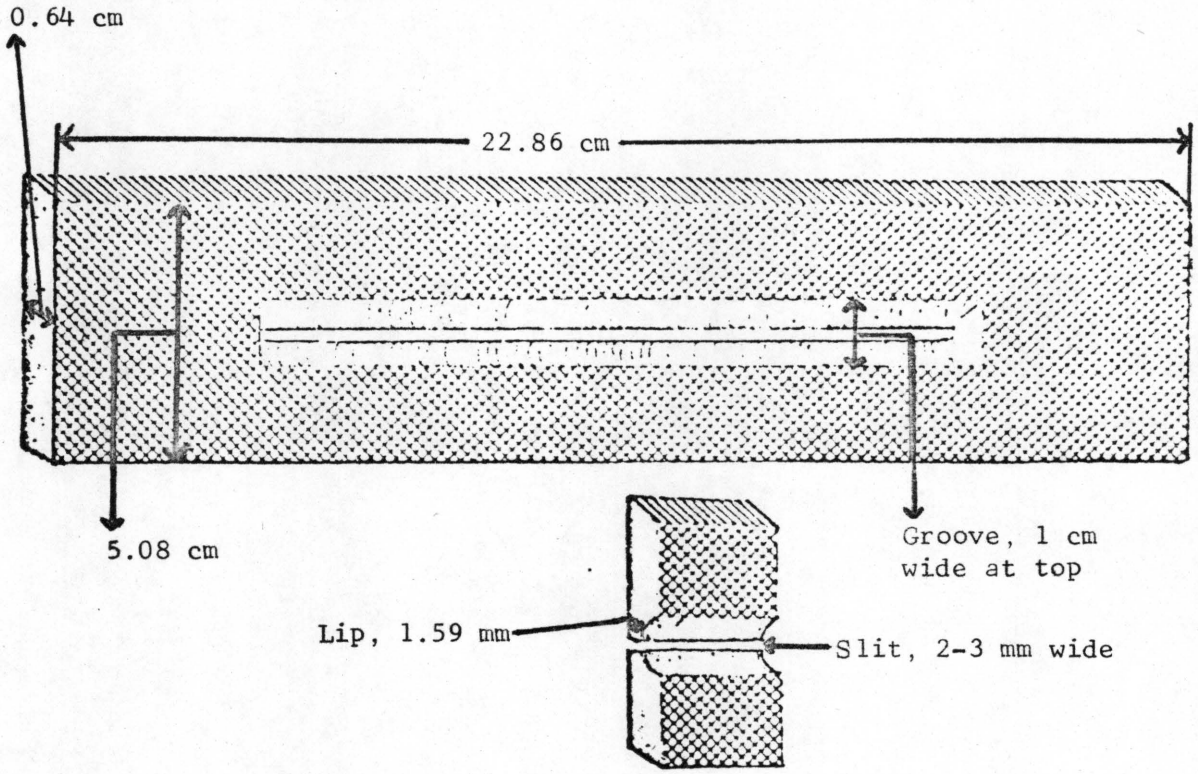


Figure 3. Adaptor for scanning polyacrylamide disc gel columns with densitometer.

cell path length = 1.00 cm; Bowen, 1949; Ledward, 1971).

The 4.5 M phosphate solution contained 393 g of K_2HPO_4 and 306 g of KH_2PO_4 in one liter of solution. It was necessary to heat the solution to 45°C with prolonged stirring to dissolve all of the solids. Some of the salt crystallized out of solution in 1-2 hours after the solution had cooled to room temperature. This solution was always heated to 45°C (with stirring) until crystallized salts were dissolved and then cooled to 35°C before use. With this treatment all of the salts were in solution at the time of use.

Investigation of the effect of "saturated" basic lead acetate solutions on determination of myoglobin concentration. The effect of "saturated" basic lead acetate solutions on determination of myoglobin concentrations was estimated from percent recovery of myoglobin in solutions containing horse heart myoglobin, bovine myoglobin and bovine sarcoplasmic proteins. The percent recovery of myoglobin in any one solution represents myoglobin that was not precipitated during the method of determination. The percent recovery of myoglobin was calculated by using a value for myoglobin concentration determined by any procedure and the following formula:

$$\frac{\text{Value for myoglobin concentration (mmole/liter)}}{\text{Total heme concentration (mmole/liter)}} \times 100 .$$

The percent recovery of myoglobin in the myoglobin-containing solutions obviously should be 100 percent, but the percent recovery of myoglobin in a solution containing bovine sarcoplasmic proteins should be only 70-80 percent. The other major heme pigment in bovine sarcoplasmic

proteins is hemoglobin (Paul, 1972a).

Myoglobin concentration was determined in solutions containing horse heart myoglobin, bovine myoglobin and bovine sarcoplasmic proteins by a modification of the Rickansrud and Henrickson (1967) procedure. "Saturated" basic lead acetate solutions[1] containing different concentrations of the compound (4-30 g per 100 ml of water) were used. A value for myoglobin concentration in solutions containing horse heart myoglobin, bovine myoglobin and bovine sarcoplasmic proteins was obtained also by a modification of the Götze (1969) procedure. Both procedures were modified by determining the absorbance of the 3 M phosphate solution at 525 nm before the cyano-derivative was prepared and that of the cyano-derivative at 540 nm. The values for myoglobin concentration determined from the absorbance of the cyano-derivative were used to calculate the percent recoveries of myoglobin and were compared with the values determined from the absorbance at 525 nm as described later in this chapter.

Total heme concentration in solutions containing horse heart myoglobin, bovine myoglobin and bovine sarcoplasmic proteins was determined by measuring the absorbance of the cyano-derivatives at 540 nm (Rickansrud and Henrickson, 1967; Götze, 1969).

"Saturated" basic lead acetate solutions were prepared from both sources of the compound. These solutions were prepared by addition

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[1]The "saturated" basic lead acetate solutions of known specific gravity that were used in the preliminary investigation also were used in this investigation.

of 4-30 g basic lead acetate per 100 ml of water and were "saturated" in the sense that an equilibrium seemed to be established between solid component and solution. The lowest level was chosen because it was below 5.55 g/100 ml water, the reported solubility of dibasic lead acetate (LIA, 1952). The highest level was chosen so that "saturated" basic lead acetate solutions would show moderate increase in specific gravity if compound(s) from either source were more soluble than dibasic lead acetate.

Basic lead acetate solutions were prepared as follows. A mixture of the compound and distilled water was stirred for one hour, and the mixture was left undisturbed for 24 hours to establish equilibrium between solution and undissolved solid. These conditions were chosen by determining that the specific gravity of the solutions did not change with further stirring or sitting, although undissolved solid was visible in each mixture. The specific gravity of the solutions was determined at 25°C and was used as an indication of the relative concentration of lead in solution (Nemedy, 1935).

A solution containing horse heart myoglobin was prepared by dispersing the powdered compound in cold water. Part of the solid matter did not dissolve and was removed from the solution by filtration through Whatman No. 42 filter paper. The total heme concentration of this solution was determined as 0.02004 mmole/liter by the previously described procedure. This solution was used in the modified procedure of Rickansrud and Henrickson (1967) and the modified procedure of Götze (1969).

Two solutions containing bovine myoglobin were prepared by diluting the concentrated solution of purified bovine myoglobin prepared as previously described with cold distilled water. One of these solutions (total heme concentration, 0.0219 mmole/liter solution) was used in the modified procedure of Rickansrud and Henrickson (1967) and the other solution (total heme concentration, 0.0342 mmole/liter solution) was used in the modified procedure of Götze (1969).

Two solutions containing bovine sarcoplasmic proteins were prepared by diluting sarcoplasmic protein extracts prepared as previously described with distilled water. One of the solutions (total heme concentration, 0.0416 mmole/liter solution) was used in the modified procedure of Rickansrud and Henrickson (1967) and the other solution (total heme concentration, 0.0490 mmole/liter solution) was used in the modified procedure of Götze (1969).

Comparison of myoglobin concentrations determined at 525 nm and at 540 nm. A t-test for paired comparison (Sokal and Rohlf, 1969) was used to analyze all values of myoglobin concentration in each myoglobin-containing solution determined from the absorbances at 525 and 540 nm by the modified procedure of Rickansrud and Henrickson (1967) and the modified procedure of Götze (1969).

Feasibility of the simplified procedure for determination of myoglobin concentration. The feasibility of the simplified procedure for the determination of myoglobin concentration was judged by determining the percent recovery of myoglobin in solutions containing horse heart

myoglobin, bovine myoglobin and bovine sarcoplasmic proteins. The percent recovery of myoglobin was calculated from the value for myoglobin concentration determined by the simplified procedure and the total heme concentration in these solutions determined from the absorbance at the isosbestic point, 525 nm (Ledward, 1971; Bowen, 1949). The average percent recovery for each myoglobin-containing solution was compared with the percent recovery found in the modified procedure of Rickansrud and Henrickson (1967) and the modified procedure of Götze (1969).

The solutions containing horse heart myoglobin, bovine myoglobin and bovine sarcoplasmic proteins were prepared as previously described except they were more concentrated. Since the simplified procedure involved a 2:1 dilution of the myoglobin-containing solution, the myoglobin-containing solutions also were diluted 2:1 with water before total heme concentration was determined from the absorbance at 525 nm.

Determination of myoglobin concentration from densitometric tracing of the electrophoretic pattern of proteins. Myoglobin concentration determined from the densitometric tracing of the electrophoretic pattern of bovine sarcoplasmic proteins was expressed as percent of total protein. It was determined by dividing the number of integrator counts under the myoglobin peak in the densitometric tracing of the electrophoretic pattern (Figure 2, page 16) by the total number of integrator counts under all of the protein peaks in the tracing. This value was compared to the value obtained for the same myoglobin

dispersion by determining myoglobin N of myoglobin (separated from other sarcoplasmic proteins) in a 3 M phosphate solution, pH 6.6, and dividing that by the total protein N in the sarcoplasmic extract. Myoglobin N and protein N were determined by a micro-Kjeldahl procedure (AOAC, 1965).

Results and Discussion

A plot of the amounts of basic lead acetate per 100 ml of water for the "saturated" basic lead acetate solutions versus the specific gravity of the solutions is shown in Figure 4. The specific gravity of the solutions increased linearly with increasing amount of added solid per 100 ml of water. This observation indicates that in the basic lead acetate from either source, part of the material is not soluble in water and part of the material is extremely soluble. Jackson (1914) investigated the equilibrium in the system of lead acetate, lead oxide and water at 25°C and reported that different solid phases were capable of existence depending on the quantity of dissolved basic lead. A "saturated" solution of basic lead acetate prepared from the sources in the present investigation is not defined in terms of the lead concentration in solution.

Shown in Table 1 are the percent recoveries of myoglobin in solutions containing horse heart myoglobin, bovine myoglobin and bovine sarcoplasmic proteins. These percent recoveries of myoglobin were determined from the values of myoglobin concentration determined in the modified procedure of Rickansrud and Henrickson (1967) and the modified procedure of Gőtze (1969). All of the percent recoveries of myoglobin

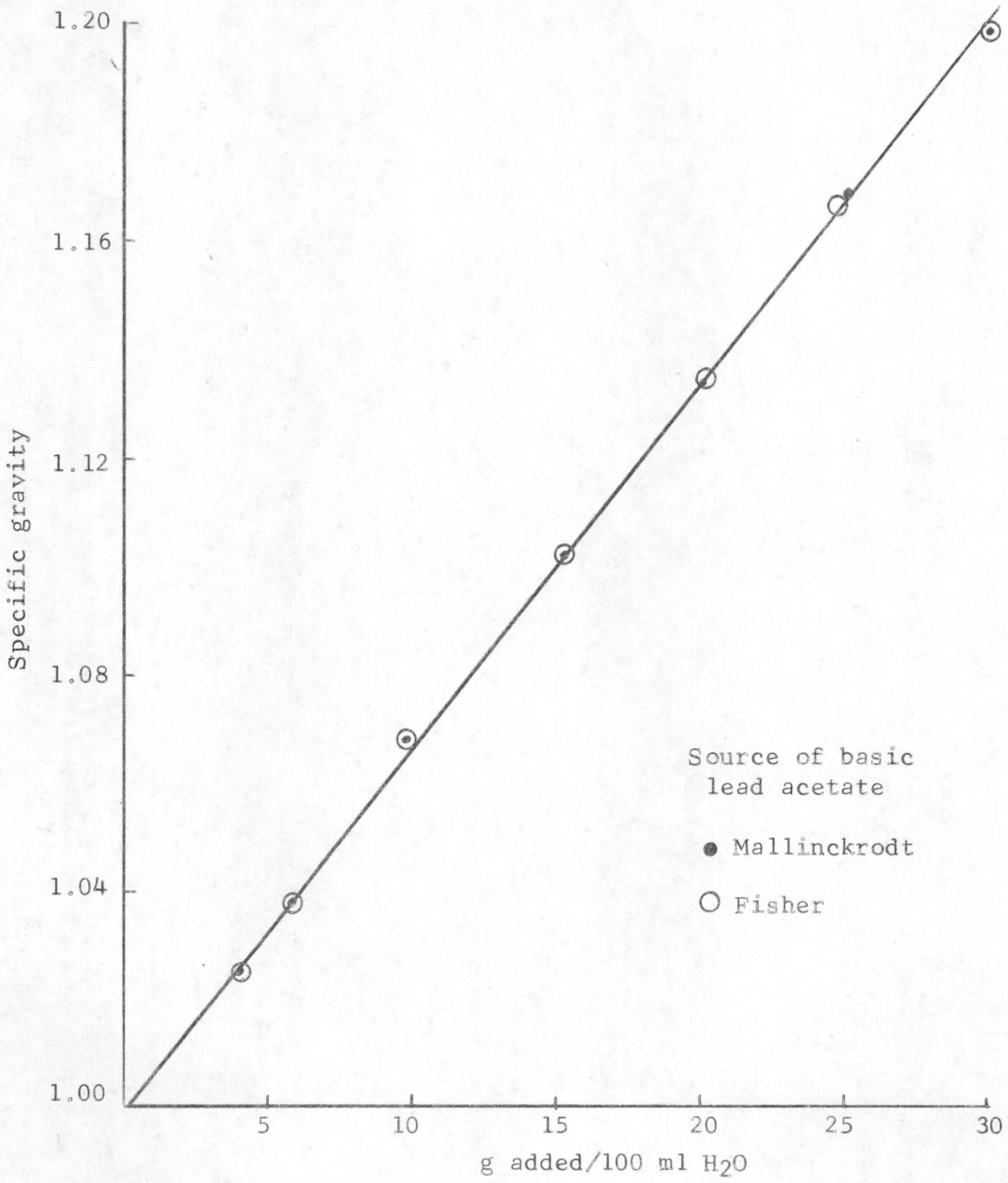


Figure 4. Plot of concentration of basic lead acetate solutions versus specific gravity of solutions at 25°C.

TABLE 1

RECOVERY OF MYOGLOBIN IN SOLUTIONS CONTAINING HORSE HEART MYOGLOBIN, BOVINE MYOGLOBIN OR BOVINE SARCOPLASMIC PROTEINS

Two Sources,[a] Basic Lead Acetate	Specific Gravity, Basic Lead Acetate Solutions, 25°C	Solution of Horse Heart Myoglobin	Solution of Bovine Myoglobin	Solution of Bovine Sarcoplasmic Proteins
		------% Recovery[b] of Myoglobin----		
	--	66.6	64.6	75.1
M	1.0258	61.9	65.7	58.2
F	1.0257	53.9	61.6	49.6
M	1.0378	50.4	65.8	45.0
F	1.0382	--	59.8	58.7
M	1.0673	51.9	65.8	51.0
F	1.0672	52.9	53.4	51.0
M	1.1022	43.4	52.5	52.2
F	1.1024	54.9	52.5	48.1
M	1.1344	42.9	53.4	38.9
F	1.1360	42.9	57.5	44.5
M	1.1676	23.5	56.6	50.0
F	1.1692	38.4	61.6	44.5
M	1.1994	36.9	54.3	41.3
F	1.1999	43.1	49.3	47.6
M	1.1745	44.9[c]	40.2[c]	45.7[c]
F	1.7892	17.0[c]	11.4[c]	21.6[c]

[a]M = Mallinckrodt; F = Fisher.

[b]Calculated from values of myoglobin concentration determined by method that eliminated use of basic lead acetate solution or by using "saturated" basic lead acetate solutions of varying specific gravity in another method.

[c]Calculated from values of myoglobin concentration determined with the "saturated" basic lead acetate solutions of the preliminary investigation.

in solutions containing horse heart myoglobin and bovine myoglobin were far below the ideal recovery of 100 percent. All of the percent recoveries of myoglobin in solutions containing bovine sarcoplasmic proteins determined in modified procedure of Rickansrud and Henrickson (1967) were below the range (70-80 percent) of myoglobin normally found in the total heme of bovine sarcoplasmic proteins (Paul, 1972a). The percent recovery of myoglobin in the solution containing bovine sarcoplasmic proteins determined in the modified procedure of Götze (1969), 75.1 percent, was in this range. The highest recoveries of myoglobin in all three myoglobin-containing solutions were found by using the modified procedure of Götze (1969) who eliminated the use of "saturated" basic lead acetate solutions. The percent recoveries determined in the modified procedure of Rickansrud and Henrickson (1967) are variable with increasing specific gravity of basic lead acetate solutions; however, the percent recoveries do tend to decrease with increasing specific gravity. The basic lead acetate solutions of specific gravity 1.1745 and 1.7892 were the solutions of "saturated" basic lead acetate that were used in the preliminary investigation and the results obtained with these solutions illustrate the problem that can arise in the use of "saturated" basic lead acetate solution without defining lead concentration in solution.

Values for myoglobin concentration determined from absorbance at 525 nm and from the absorbance of cyano-metmyoglobin at 540 nm in the modified procedure of Rickansrud and Henrickson (1967) and the modified procedure of Götze (1969) are shown in Table 2. A t-test for paired

TABLE 2

CONCENTRATIONS OF MYOGLOBIN IN SOLUTIONS CONTAINING HORSE HEART MYOGLOBIN, BOVINE MYOGLOBIN OR BOVINE SARCOPLASMIC PROTEINS DETERMINED BY ABSORBANCE AT 525 nm AND BY ABSORBANCE OF CYANOMETMYOGLOBIN AT 540 nm

Specific Gravity Lead Acetate	Bovine Myoglobin		Horse Heart Myoglobin		Sarcoplasmic Protein	
	525 nm	540 nm	525 nm	540 nm	525 nm	540 nm
	Millimole/Liter					
1.0258	0.0161	0.0154	0.0134	0.0124	0.0241	0.0242
1.0257	0.0147	0.0144	0.0107	0.0108	0.0252	0.0248
1.0378	0.0141	0.0154	0.0107	0.0101	0.0199	0.0187
1.0382	0.0132	0.0140	--	--	0.0252	0.0244
1.0673	0.0147	0.0154	0.0101	0.0104	0.0218	0.0212
1.0672	0.0121	0.0125	0.0105	0.0106	0.0225	0.0212
1.1022	0.0132	0.0123	0.0091	0.0087	0.0208	0.0217
1.1024	0.0121	0.0123	0.0114	0.0110	0.0199	0.0200
1.1344	0.0121	0.0125	0.0080	0.0086	0.0161	0.0162
1.1360	0.0154	0.0135	0.0083	0.0086	0.0193	0.0185
1.1676	0.0132	0.0133	0.0054	0.0047	0.0194	0.0208
1.1692	0.0161	0.0144	0.0064	0.0077	0.0174	0.0185
1.1994	0.0132	0.0127	0.0064	0.0074	0.0172	0.0172
1.1999	0.0114	0.0156	0.0072	0.0088	0.0188	0.0198
1.1745	0.0094	0.0094	0.0093	0.0090	0.0188	0.0190
1.7892	0.0027	0.0027	0.0034	0.0034	0.0087	0.0090
--	0.0221[a]	0.0219[a]	0.0141[a]	0.0149[a]	0.0368[a]	0.0345[a]

[a]These values were determined by the modified procedure of U. Gőtze. 1969. Determination of myoglobin and haemoglobin in meat extract from slaughtered animals. Fleischwirtschaft 49: 901. Cited in Food Sci. Technol. Abstracts 2: 2S92.

comparisons between these values of myoglobin concentration for each source of myoglobin showed that there was no significant difference between the concentration values as determined at 525 nm and at 540 nm.

The percent recoveries of heme pigment in solutions containing horse heart myoglobin, bovine myoglobin and bovine sarcoplasmic proteins determined by the simplified procedure are shown in Table 3. The percent recoveries determined in triplicate for any one solution agree reasonably well. The average percent recovery of myoglobin in any one solution was higher than the percent recovery of myoglobin in a solution of the same kind determined in the modified procedure of Götze (1969). The average percent recoveries of myoglobin in the solutions containing horse heart myoglobin and bovine myoglobin were still below the ideal recovery of 100 percent, but the average percent of recovery of myoglobin in the solution containing bovine sarcoplasmic proteins was still in the range (70-80 percent) of myoglobin normally found in the total heme of bovine sarcoplasmic proteins (Paul, 1972a). Since the highest percent recoveries of myoglobin in pure myoglobin-containing solutions were found by this simplified procedure, and since the percent myoglobin in total heme (percent recovery of myoglobin) of bovine sarcoplasmic proteins found by this procedure was in the range previously reported, it was decided that this simplified procedure was indeed feasible.

Myoglobin content (expressed as percent of total protein) in solutions containing bovine sarcoplasmic proteins is shown in Table 4. These values were determined from densitometric tracings of the electrophoretic pattern of bovine sarcoplasmic proteins (Figure 2, page 16)

TABLE 3

PERCENT RECOVERY OF MYOGLOBIN IN SOLUTIONS CONTAINING HORSE HEART MYOGLOBIN, BOVINE MYOGLOBIN OR BOVINE SARCOPLASMIC PROTEINS DETERMINED BY USE OF THE SIMPLIFIED PROCEDURE FOR DETERMINATION OF MYOGLOBIN CONCENTRATION

Replication Number	Horse Heart Myoglobin	Bovine Myoglobin	Bovine Sarcoplasmic Protein
	% Recovery		
1	71.8	88.9	79.4
2	70.4	85.9	77.9
3	71.3	85.9	78.8
Average	71.2	86.9	78.7

TABLE 4

MYOGLOBIN CONTENT (EXPRESSED AS PERCENT OF TOTAL PROTEIN) IN SOLUTIONS CONTAINING BOVINE SARCOPLASMIC PROTEINS DETERMINED FROM DENSITOMETRIC TRACINGS OF ELECTROPHORETIC PATTERN OF THE PROTEINS AND FROM MICRO-KJELDAHL ANALYSIS

	Tracings of Electrophoretic Pattern			Micro-Kjeldahl		
	Integrator Counts			Mg N/Ml Solution		
Sample	Myoglobin Peaks	All Protein Peaks	Myoglobin Content	Myoglobin	Total Protein	Myoglobin Content
			% of Total Protein			% of Total Protein
1	830	3360	24.7	0.428	2.259	18.9
2	1150	4520	25.4	0.449	2.288	19.6
3	1009	3870	26.1	0.444	2.228	19.9

and from micro-Kjeldahl analysis. The percentages determined from the tracing of the electrophoretic pattern are consistently higher than those found by micro-Kjeldahl analysis.

Kruski and Narayan (1968) in an investigation of some quantitative aspects of polyacrylamide disc gel electrophoresis using densitometric tracings found: (1) that there was a linear relationship between total peak area and amounts of protein only up to 15 µg of protein and (2) that the total peak area became larger as the protein was allowed to penetrate farther into the gel. They state that when dealing with biological mixtures of proteins such as serum proteins it is not possible to preset sample size or penetration distance of all components to their optimum range and that the components present in larger amounts and components of high molecular weight that do not penetrate very far will appear to be present in smaller proportion of the mixture than they really are.

In the densitometric tracing of the electrophoretic pattern in Figure 2, page 16, peak 1 is a broad band that did not penetrate far into the gel. This peak probably represents proteins of high molecular weight which appeared to be present in a smaller percentage of total proteins than they really were. It is also possible that myoglobin was allowed to penetrate too far into the gel so that the total myoglobin peak area was increased out of proportion to the actual myoglobin content. Both of these factors would cause the myoglobin content determined from the tracing and expressed as percent of total protein to be greater than it really should be.

Another factor, on the other hand, could cause the value for myoglobin content to be less than it should be. Approximately 140 µg of bovine sarcoplasmic proteins were separated by disc gel electrophoresis to give the electrophoretic pattern which was traced by the densitometer (Figure 2, page 16). Multiplying the average myoglobin content in total protein determined by micro-Kjeldahl analysis (19.5 percent) by the total weight of proteins represented in Figure 2 shows that the myoglobin peaks represent approximately 27 µg of myoglobin, an amount that is beyond the range of linearity reported by Kruski and Narayan (1968). This would cause the myoglobin content to appear to be less than it really was.

All three factors in combination must account for the difference found between values for the myoglobin contents determined by two methods and shown in Table 4, page 30. It is apparent that it is difficult to determine quantitatively the content of one protein in a mixture of different molecular weight proteins using polyacrylamide disc gel electrophoresis.

Summary

Myoglobin concentrations were determined in solutions containing horse heart myoglobin, bovine myoglobin and bovine sarcoplasmic proteins by using "saturated" basic lead acetate solutions containing different amounts of basic lead acetate per 100 ml of water in a modified procedure of Rickansrud and Henrickson (1967). "Saturated" basic lead acetate did not define lead concentration in solution. Higher recoveries

of myoglobin were obtained in solutions containing horse heart myoglobin or bovine myoglobin by a modified procedure of Götze (1969) who eliminated the use of "saturated" basic lead acetate solutions. Significant differences did not exist between myoglobin concentrations determined from the myoglobin isosbestic point at 525 nm and from the cyanometmyoglobin peak at 540 nm.

Highest recoveries of myoglobin were obtained in solutions containing horse heart myoglobin or bovine myoglobin by a proposed simplified procedure for the determination of myoglobin concentration. This procedure involved: (1) the addition of a 4.5 M phosphate solution to a myoglobin-containing solution (such as a water extract of bovine muscle) in the ratio of 2:1 v/v resulting in a 3 M phosphate solution containing only myoglobin and (2) the determination of the concentration of the myoglobin from absorbance at the isosbestic point of all forms of myoglobin, 525 nm.

Efforts to use densitometric tracings of the polyacrylamide disc gel electrophoretic pattern of bovine sarcoplasmic proteins to determine myoglobin content in a solution of such proteins were unsuccessful.

CHAPTER III

PROTEIN-PHOSPHOLIPID INTERACTIONS IN HEATED PHOSPHOLIPID EMULSIONS CONTAINING BOVINE MYOGLOBIN OR SARCOPLASMIC PROTEINS.

I. PROTEIN SOLUBILITY, POSSIBLE REACTION SITES AND ELECTROPHORETIC PATTERNS

Introduction

Myoglobin reacts to form brown insoluble pigments in cooked meat and this reaction has been the subject of numerous investigations. Several investigators (Bernofsky et al., 1959; Siedler and Schweigert, 1959; Draudt, 1969) have shown that myoglobin precipitates in meat at 60°C and above, but that in pure solution at the pH of meat, marked precipitation occurs only at temperatures above 75°C.

Draudt (1969) found that precipitation of myoglobin in meat was dependent on the nature of the ligand at the sixth position and on the oxidation state of the iron. Tappel (1957) established that the iron in the denatured heme compounds is in the iron(III) state. The ligand at the fifth position may be denatured globin, the protein part of myoglobin (Tappel, 1957; Tarladgis, 1962; Brill and Williams, 1961; Hollocher, 1966).

Draudt (1969) suggested that myoglobin apparently co-precipitates with other proteins in meat. Thus, part of the denaturation of myoglobin is attributed to denaturation of these proteins in meat. Ledward (1971) investigated the role of water-soluble proteins in the formation

of cooked meat hemoprotein and suggested that the protein in the denatured hemoprotein may be any of several of the denatured proteins in cooked meat. His results indicated that precipitation of myoglobin in aqueous muscle extracts, pH 6.6 heated to 60-65°C, was caused by higher molecular weight protein. Atanasov and Mitova (1970) suggested that proteins could interact with globin (the protein part of myoglobin) destabilizing the globin and causing "normal" denaturation at a lower temperature. It is possible that suitable sites of the denatured proteins could form linkages with the sides of the hematin (e.g., the carboxylate groups) as with methemalbumin (O'Hagan, 1959) again causing myoglobin to denature at a lower temperature.

The investigations that implicated myoglobin-protein interactions in myoglobin denaturation in meat did not involve myoglobin interactions with nonprotein muscle constituents. There is a possibility that other constituents such as lipids could interact with myoglobin and these interactions could protect or destabilize myoglobin to heat denaturation.

Reports were not found in which interactions between lipids and muscle proteins were identified or measured in cooked meats or in heated reaction systems. Campbell and Turkki (1967) showed that drip phospholipids from cooked beef consist largely of cephalins and that the cooked meat contains a significantly lower cephalin concentration than the raw meat. These observations suggest a partitioning of phospholipid classes during the cooking of meat, and it is possible that interactions of phospholipids with water-soluble muscle constituents during heating could cause this partitioning.

There are numerous reports of phospholipid interactions with other proteins. Fullington (1969) reported many types of interactions between lipids and flour dough proteins. These interactions include hydrophobic, ionic and hydrogen bonding and mixed chelate formation. Ikeda and Taguchi (1968) found that the solubility decrease of fish actomyosin during storage at 2°C was lessened with addition of lecithin. Phospholipids also have been shown to bind with soybean proteins (Rzhekhin and Krasilnov, 1965).

Polyacrylamide disc gel electrophoresis was used by Laakkonen et al. (1970) to detect changes in bovine water soluble proteins during heating. Other investigators (Quinn et al., 1964; Quinn and Pearson, 1964) showed that the bovine myoglobin, which was separated into three major bands by starch-gel electrophoresis was not affected by heating to 55°C for 5 minutes. Höyem and Thorson (1970) reported that polyacrylamide disc gel electrophoresis could be used to identify meat from different animal sources (such as whale, horse and beef) on the basis of migration of the soluble myoglobin as bands in the gel, but they were unable to obtain an electrophoretogram of myoglobin in heated products. It would seem, therefore, that disc gel electrophoresis could be a useful tool in an investigation of denaturation of bovine water-soluble proteins, especially myoglobin, in heated systems.

This study was initiated to use polyacrylamide disc gel electrophoresis as a tool in the investigation of possible interactions between phospholipids and bovine myoglobin, either isolated or as a component of sarcoplasmic proteins, and between phospholipids and total sarcoplasmic proteins.

Materials and Methods

Bovine proteins and lipids. Purified bovine myoglobin and bovine sarcoplasmic proteins were prepared by the methods described in Chapter II. Sarcoplasmic protein extracts were used within 48 hours of extraction from meat. The meat was from a homogeneous mixture of bovine ground round which was stored in aluminum foil wrapped packages of 100 g at -20°C.

Lipids were extracted from bovine ground round by the method of Bligh and Dyer as modified by Ostrander and Dugan (1961). Phospholipids were separated from neutral lipids on a silicic acid column by Campbell and Turkki's (1967) modification of the Hornstein et al. (1961) method. Enough lipids to complete all of the planned investigations were prepared and pooled before experimentation started. The pooled phospholipids were stored under nitrogen at -20°C in a chloroform solution.

Equipment. All spectrophotometric analyses were done on a Bausch and Lomb Spectronic 20 colorimeter, a direct-reading grating spectrophotometer, with a circular cell of 1.17 cm light path length. All centrifugation was done on a Lourdes Model A-2 Beta-fuge, refrigerated centrifuge, with a 9RA head. All pH measurements were made on a Beckman Zeromatic pH meter.

Preparation of protein-containing solutions. The solution containing sarcoplasmic protein that was prepared according to the method described on page 13 had a protein concentration of 14 mg/g solution

as determined by micro-Kjeldahl analysis and use of 6.25 as the conversion factor of mg protein nitrogen to mg sarcoplasmic protein. Myoglobin-containing solutions prepared by diluting the purified bovine myoglobin (page 11) had a concentration of approximately 2 mg/g solution as determined by micro-Kjeldahl analysis and using 5.98 as the conversion factor for mg protein N to mg myoglobin. This factor was calculated from the amino acid composition of bovine myoglobin reported by Atassi et al. (1970).

Cold samples (1°C) of the protein-containing solution to be heated (not over 105 g) were blended for 30 seconds in a micro jar on a Waring blendor at a setting of 90 on a Powerstat variable transformer. Longer times or higher speeds of blending caused excessive foaming. Protein-containing solutions were blended to subject the proteins in the solutions to the same type of mechanical stress that the proteins underwent in the making of the emulsions.

Preparation of protein-lipid emulsions. Protein-phospholipid emulsions were prepared as follows. An aliquot of the phospholipid solution in chloroform was dried under a stream of nitrogen at room temperature. As quickly as possible dried phospholipids were added to a cold aqueous protein-containing solution (0.8 g phospholipids/100 g solution) in small portions and the mixture was emulsified by blending 30 seconds in a micro jar on a Waring blendor at a setting of 90 on a Powerstat variable transformer. The blending was done in 10-sec intervals. After each interval the sides of the micro jar were scraped down with a stainless steel spatula. A nitrogen atmosphere

was maintained over the phospholipids at all times.

Two different protein-phospholipid emulsions were prepared: myoglobin-phospholipid and sarcoplasmic protein-phospholipid. Emulsions were prepared separately for 25°C and the different end point temperatures and enough of each sample was prepared at one time to do all of the planned analyses. Protein concentration in the sarcoplasmic protein-lipid emulsion and in the myoglobin-lipid emulsion was the same as that in the corresponding protein-containing solutions.

Heating of protein-containing solutions and protein-lipid emulsions. Solutions containing sarcoplasmic proteins and sarcoplasmic protein-phospholipid emulsions were heated under a nitrogen atmosphere from 25°C to end point temperatures of 56, 60, 65 and 77°C at a rate of temperature increase of 1°C/min in a water bath set-up shown in Figure 5. Myoglobin-containing solutions and myoglobin-phospholipid emulsions were heated from 25°C to end point temperatures of 65 and 77°C at the same rate and in the same apparatus. The solutions[2] and emulsions were evaluated at 25°C and at each end point temperature.

It was possible to heat at one time three samples of a solution or an emulsion to different end point temperatures. The heating of each solution or emulsion to each end point temperature was duplicated, and the duplicates of each end point temperature for a particular

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[2]When a solution is mentioned in this chapter, it refers to a solution containing dispersed proteins rather than a "true" solution of proteins.

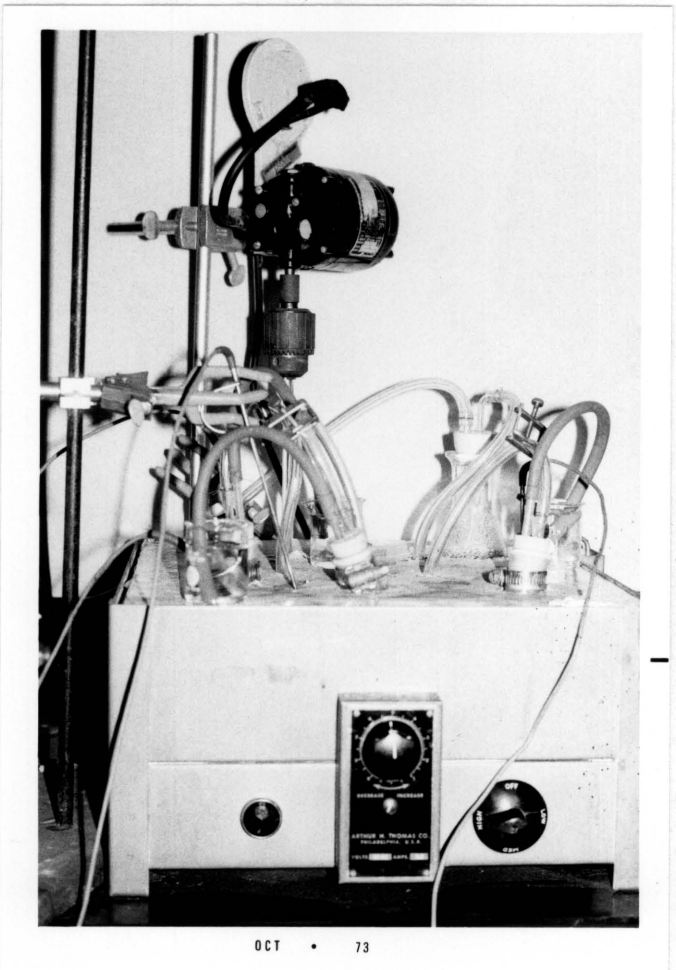


Figure 5. Water bath containing apparatus that was used in heating dispersion from 25°C to different end point temperatures up to 77°C.

solution or emulsion were always in different heating groups.

For each end point temperature a weighed sample of an emulsion or solution was placed in a 250-ml round bottom flask with a thermometer well. Three flasks were suspended in the water bath (Figure 5) which had been set to increase the temperature from 25°C to the end point temperatures at the desired rate. A thermocouple was inserted in each flask and the temperatures were monitored by a Honeywell temperature recorder during heating. Nitrogen was scrubbed with distilled water the same temperature as the water bath and was bubbled through each flask at a flow rate of 10-20 ml/min. When a flask reached the desired end point temperature, it was removed from the water bath and cooled in an ice bath to 25°C. The amount of evaporation that occurred during heating was determined gravimetrically and the flask contents were saved for further evaluation.

Evaluation of protein-containing solutions. Upon attainment of each end point temperature and cooling, the sample was centrifuged at 18°C for 15 minutes at 25,500 x g to separate the heat-precipitated protein and the protein remaining dispersed in solution.[3] After centrifugation, the heat-precipitated protein was washed from the centrifuge tube and made into a slurry by mixing it with 9 ml of distilled water in a mortar and pestle. The following measurements were made on unheated (25°C) samples and on the slurried precipitate and the

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[3]In the heated myoglobin-containing solutions no precipitate formed until the pH of the heated solution was adjusted to 7.00.

supernatants from the heated samples: (1) protein content by micro-Kjeldahl analysis (AOAC, 1965);[4] (2) available lysine by the method of Kakade and Liener (1969); and (3) basic and acidic groups by the method of Hamm and Deatherage (1960). The pH of the solution at 25°C and at each end point temperature was measured and the electrophoretic pattern of the "soluble" proteins was determined for the same temperatures according to the procedure given on page 13. Myoglobin concentration was determined in all protein-containing solutions at 25°C and at each end point temperature by the simplified procedure given on page 15. Total heme protein was determined in all protein-containing solutions by the method of Ledward (1971).

Evaluation of protein-lipid emulsions. Emulsions heated to the end point temperatures were treated in the manner described for the heated solution except that a nitrogen atmosphere was maintained over all phospholipid-containing mixtures. Figures 6 and 7 are schematic flow charts for the evaluation of protein-lipid emulsions at 25°C and after heating, respectively. Evaluation of the free and bound lipid content and phospholipid classes is discussed in Chapter IV. All measurements were made by the methods described in reference to protein-containing solutions.

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[4]Protein content of myoglobin-containing and sarcoplasmic protein-containing solutions was determined at the unadjusted pH of the dispersion (measured pH) and after pH adjustment to 7.00 with phosphate buffer, 0.5 M, pH 7.1.

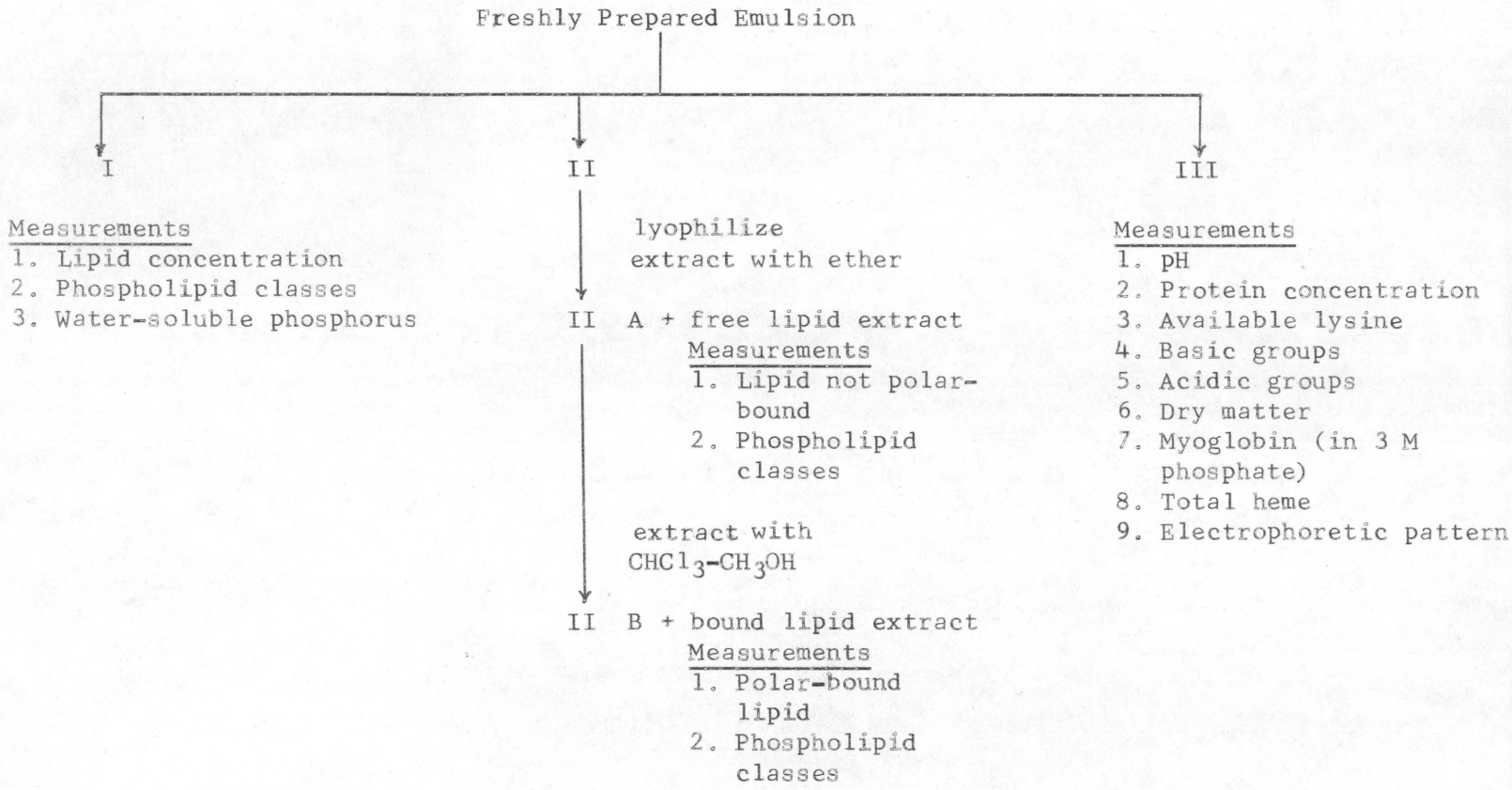


Figure 6. Evaluation of protein-phospholipid emulsions at 25°C.

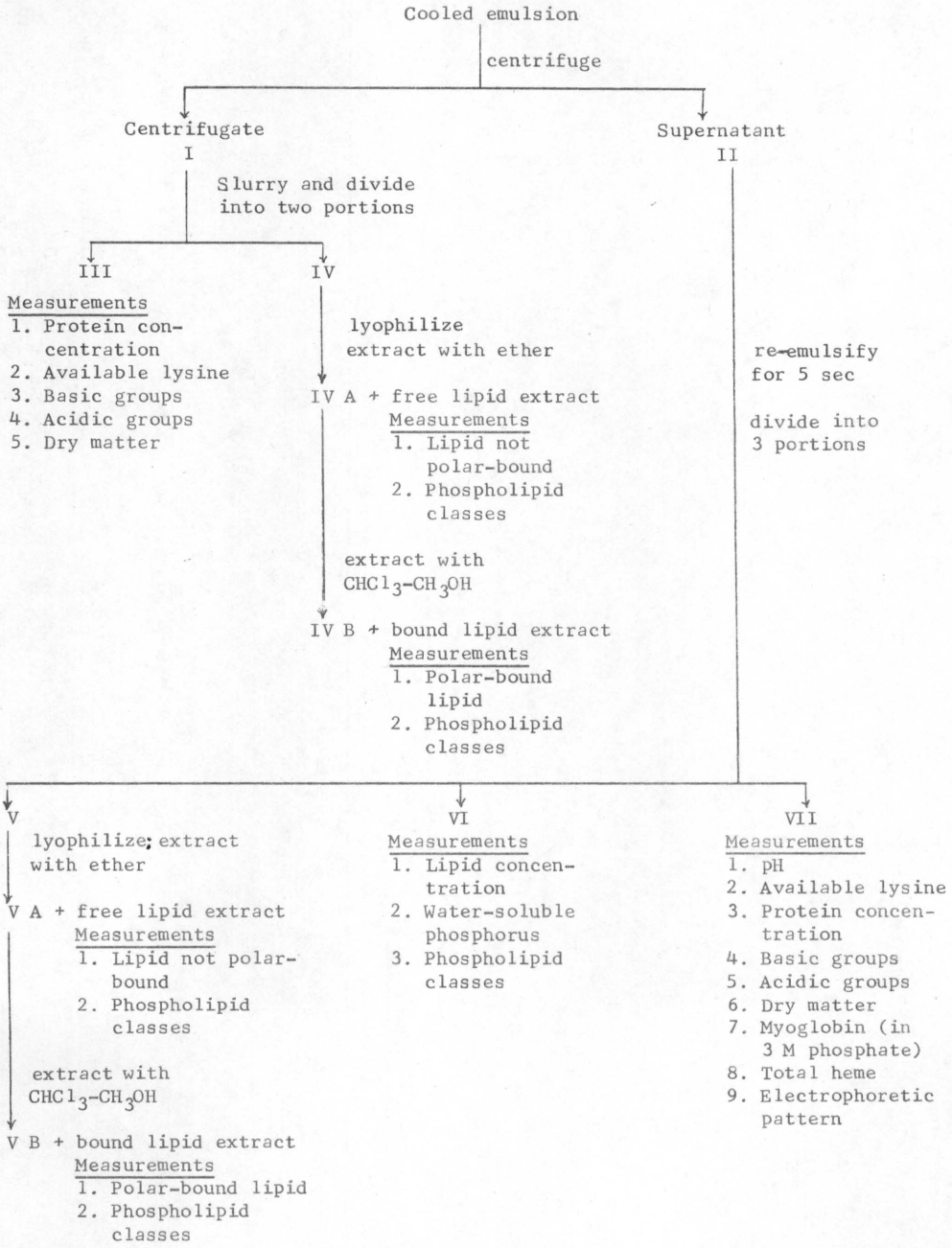


Figure 7. Evaluation of protein-phospholipid emulsions at end point temperatures.[a]

[a]Lipid concentration, water-soluble phosphorus and phospholipid classes were determined on an aliquot of each emulsion before heating.

Mathematical and statistical procedure. The weight-fraction of protein that remained dispersed at each end point temperature in solution or emulsion was determined by dividing the total mg protein remaining dispersed in the heated sample by the total mg protein dispersed in the sample before heating. The weight-fraction precipitated was 1.000 minus the fraction of dispersed protein. These fractions were used to determine changes caused by heat in such measurements as available lysine, acidic groups and basic groups for total protein in the heated sample according to the following equation:[5]

$$\text{Weight-fraction, precipitated protein} \left(\frac{\text{measurement}}{\text{10 kg precipitated protein}}\right) +$$

$$\text{weight-fraction, dispersed protein} \left(\frac{\text{measurement}}{\text{10 kg dispersed protein}}\right) = \left(\frac{\text{measurement}}{\text{10 kg total protein}}\right).$$

Two way analysis of variance with replication for a Model I Anova was used for the analysis of all data. The Student-Newman-Keuls test was used to identify significant differences between means. All statistical procedures were carried out according to Sokal and Rohlf (1969).

Results and Discussion

Protein remaining dispersed in heated protein-containing solutions and in heated protein-lipid emulsions, as determined by Kjeldahl analysis. The percent of sarcoplasmic protein remaining dispersed in

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[5]In the case of myoglobin-containing solutions or emulsions the measurements were expressed per mole myoglobin instead of per 10 kg protein.

heated sarcoplasmic protein dispersions at the measured pH[6] (5.31-5.69) and after pH adjustment to 7.00 is given in Table 5. In the protein-containing solution at the measured pH the amount of dispersed protein decreased significantly with each increase in temperature except between the temperatures of 60 and 65°C. In the emulsion at the measured pH heating from 25 to 56°C caused a significant decrease in percent dispersed protein but heating to higher temperatures had no effect. In dispersions heated to 65 and 77°C significantly more protein remained dispersed in the emulsion than in the solution. The significant temperature-dispersion interaction occurred between 56 and 60°C. At 56°C more protein remained dispersed in the solution than in the emulsion; at 60°C the reverse was true. These facts indicate an interaction between phospholipids and sarcoplasmic proteins at the measured pH that protected the sarcoplasmic proteins from heat precipitation above 56°C.

When the pH was adjusted to 7.00 in solutions and emulsions that had been heated, additional protein precipitated in both dispersions for all heating temperatures. Significant decreases in percent protein dispersed in solutions that were adjusted to pH 7.00 after heating were found with each increase in temperature. Significant decreases in percent protein dispersed in the heated emulsion adjusted to pH 7.00 also were observed with each increase in temperature except between 56

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[6]Used here to refer to unadjusted dispersion pH, as measured after heating.

TABLE 5

PERCENT PROTEIN DISPERSED IN HEATED SOLUTIONS CONTAINING SARCOPLASMIC PROTEINS AND IN HEATED SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS AT THE MEASURED pH AND THE ADJUSTED pH OF THE DISPERSIONS

Dispersion[g]	Temperature[g] to Which Dispersions Were Heated				
	25°C	56°C	60°C	65°C	77°C
	Measured pH (5.31-5.69)				
	%				
Solution	100.0[a]	69.2[b]	56.7[c]	48.2[c]	42.6[d]
Emulsion	100.0[a]	62.1[b]	63.5[b,c]	64.0[b]	60.4[b]
	Adjusted pH (7.00)				
	%				
Solution	89.8[a]	63.3[c]	48.0[d]	29.5[e]	9.8[f]
Emulsion	79.8[b]	49.7[d]	43.6[d]	27.1[e]	15.3[f]

[a,b,c,d,e,f]For either the measured pH or the adjusted pH, percentages in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

[g]The temperature-dispersion interactions for the measured pH and the adjusted pH are significant at the $P < 0.01$ level.

and 60°C. In 25 and 56°C samples the percent protein dispersed in the emulsion at pH 7.00 was significantly less than in the solution at pH 7.00 but no significant differences were found at 60, 65 and 77°C. The significant temperature-dispersion interaction at pH 7.00 shows that while percent protein dispersed in both dispersions decreased with increasing temperature, the percent protein dispersed in the emulsion decreased less than did the percent protein dispersed in the solution. All of these observations indicate interactions between phospholipids and sarcoplasmic proteins in emulsions to form complexes which after heating are less stable to higher pH (7.00) but somewhat more stable to heat-precipitation than the proteins alone.

Several investigators have measured sarcoplasmic (water-soluble) proteins in cooked beef, rabbit and pork (Hamm and Deatherage, 1960; Cohen, 1966; Paul et al., 1966; Randall and Bratzler, 1970; and Laakkonen et al., 1970). Cohen (1966) noted that the amount of protein altered during heating was a function of time and temperature. The above investigations were conducted with various time-temperature combinations and it would not be valid to compare the results of the earlier investigations with these of the present investigation. It is generally agreed, however, that sarcoplasmic (water-soluble) proteins start to precipitate in meat between 40 and 60°C and that most of the proteins are denatured by 62°C (Bendall, 1964). Bendall further noted that the nature of the coagulum depended on pH and ionic strength of the system.

Reports were not found of investigations of interactions between bovine sarcoplasmic proteins and phospholipids.

The percent myoglobin dispersed in heated myoglobin dispersions at the measured pH (4.07-5.71) and at the adjusted pH (7.00) is reported in Table 6. No decrease with increasing temperature was found for myoglobin dispersed in solutions at the measured pH. Differences in percent myoglobin dispersed in the emulsion at the measured pH between end point temperatures appear large but were not significant, probably because the mean value reported at 65°C is an average of two widely different values (65.0 and 95.5). In emulsions heated to 65 and 77°C the percent myoglobin dispersed at the measured pH was significantly smaller than that in the solution. When the pH of the dispersions that had been heated was adjusted to 7.00, additional protein precipitated in all solutions and emulsions. At pH 7.00 the apparent effect of temperature on dispersed protein in solution was not significant. A significant difference was found between percent myoglobin dispersed in emulsions heated to 77°C and for emulsions heated to all other temperatures. For each temperature in dispersions of pH 7.00 significantly less myoglobin remained dispersed in the emulsion than in the solution. These results indicate the formation of a myoglobin-phospholipid complex that is less stable to heat-precipitation than myoglobin and after heating is less stable to higher pH (7.00) than the heated myoglobin alone.

Total heme protein and myoglobin remaining dispersed in heated sarcoplasmic protein-lipid emulsions. The percent total heme protein and percent myoglobin dispersed in sarcoplasmic protein dispersions are reported in Table 7. Significant decreases were observed with

TABLE 6

PERCENT MYOGLOBIN DISPERSED IN HEATED SOLUTIONS CONTAINING MYOGLOBIN AND IN HEATED MYOGLOBIN-PHOSPHOLIPID EMULSIONS AT THE MEASURED pH AND THE ADJUSTED pH OF THE DISPERSIONS

Dispersion	Temperatures to Which Dispersions Were Heated		
	25°C	65°C	77°C
	Measured pH (4.07-5.71)		
	%		
Solution	100.0[a]	100.0[a]	100.0[a]
Emulsion	100.0[a,b]	80.2[b]	76.5[b]
	Adjusted pH (7.00)		
	%		
Solution	97.1[a]	86.1[a]	80.3[a]
Emulsion	78.8[b]	65.5[b]	52.8[c]

[a,b,c]For either the measured pH or the adjusted pH, percentages in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

TABLE 7

PERCENT TOTAL HEME PROTEIN AND PERCENT MYOGLOBIN DISPERSED IN HEATED SOLUTIONS CONTAINING SARCOPLASMIC PROTEINS AND IN HEATED SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

Dispersion[f]	Temperatures[f] to Which Dispersions Were Heated				
	25°C	56°C	60°C	65°C	77°C
	Total Heme Protein				
	%				
Solution	100.0[a]	92.5[b]	90.1[b,c]	85.9[c]	37.8[d]
Emulsion	100.0[a]	85.9[e]	81.8[e,f]	78.9[f]	42.0[d]
	Myoglobin				
	%				
Solution	100.0[a]	102.4[a]	100.8[a]	98.8[a]	39.8[b]
Emulsion	100.0[a]	101.2[a]	101.1[a]	94.0[b]	45.3[c]

[a,b,c,d,e]Percentages either for dispersed total heme protein or dispersed myoglobin in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

[f]The temperature-dispersion interaction for percent dispersed total heme protein and percent dispersed myoglobin is significant at the $P < 0.05$ level.

increasing temperature for percent total heme protein dispersed in solution. The significant decreases were observed between 25°C and each end point temperature and between all additional pairs of temperatures except between 56 and 60°C and between 60 and 65°C. Significant decreases also were observed with increasing temperature for percent total heme protein dispersed in the emulsions with the same exceptions. At 56, 60 and 65°C, percent total heme protein dispersed in the emulsion was less than that in the solution. At 77°C more heme protein remained dispersed in the emulsion than in the solution but the difference was not significant. The significant temperature-dispersion interaction for total heme protein suggests that although a heme protein-phospholipid complex is significantly less stable than is the heme protein to heat precipitation at temperatures of 56, 60 and 65°C, the complex may be more stable at 77°C (Table 7).

The only significant decrease found at higher temperatures for the percent myoglobin[7] remaining dispersed in solutions was between 77°C and all other temperatures. Significant decreases observed with higher temperatures for percent myoglobin dispersed in the emulsion were found after the emulsions were heated past 60°C. Significantly less myoglobin remained dispersed in the emulsion than in the solution at 65°C but the reverse was true at 77°C. The significant temperature-dispersion interaction indicates possible existence of a myoglobin-

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[7]Percents higher than 100 were observed because the absorbance of the isolated myoglobin in 3 M phosphate at 525 nm was greater after heating than it was before heating.

phospholipid-protein complex that has less stability to heat-precipitation at 65°C and more stability to heat precipitation at 77°C than has a myoglobin-protein complex.

Myoglobin (as determined by simplified procedure) remaining dispersed in heated protein-lipid emulsions. The percent myoglobin dispersed in myoglobin dispersions and in sarcoplasmic protein dispersions is reported in Table 8. The percent myoglobin dispersed in the solution at 25°C was considerably less than that (86-88 percent, Table 3, page 29) in a solution that was not subjected to the blending procedure. This difference indicates that some changes must have occurred in the molecular structure of part of the myoglobin in the blending process that altered the solubility in 3 M phosphate solutions. As was seen for the results obtained by Kjeldahl analysis (page 49), significant decreases were not observed at increasing temperatures for percent myoglobin dispersed in solution.

Nearly all of the myoglobin in the myoglobin emulsion at 25°C precipitated in 3 M phosphate solution. This suggests that myoglobin was complexed with phospholipids in such a way that either the hydration or charges of the myoglobin molecule were altered so that it precipitated in 3 M phosphate solution.

The myoglobin molecule in aqueous dispersion was reported to have a highly polar surface which interacts with water molecules and a non-polar, hydrophobic interior which appears to contribute much to the stability of the whole molecule (Jones, 1964). There is general agreement among investigators (Barratt et al., 1968; Fullington, 1969;

TABLE 8

PERCENT MYOGLOBIN[f] DISPERSED (AFTER 3 M PHOSPHATE ISOLATION) IN THE FOLLOWING HEATED DISPERSIONS: MYOGLOBIN-CONTAINING SOLUTIONS, MYOGLOBIN-PHOSPHOLIPID EMULSIONS, SOLUTIONS CONTAINING SARCOPLASMIC PROTEINS, SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

Type Dispersion[g]	Temperatures[g] to Which Dispersions Were Heated		
	25°C	65°C	77°C
Myoglobin			
Solution	69.4[a]	67.6[a]	64.8[a]
Emulsion	1.8[b]	27.4[c]	38.9[d]
Sarcoplasmic			
Solution	100.0[e]	98.8[e]	39.8[d]
Emulsion	100.0[e]	94.0[e]	45.3[d]

[a,b,c,d,e]Percentages in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

[f]See Simplified procedure for determination of myoglobin concention, page 15.

[g]The temperature-dispersion interaction is significant at the $P < 0.001$ level.

Gulik-Krzywicki et al., 1969) that interactions of phospholipids with proteins are polar in nature. Interactions that change the hydration of the myoglobin molecule, therefore, could cause its precipitation in more concentrated salt solutions where it is normally soluble.

Significant increases were observed with increasing temperature for percent myoglobin dispersed in myoglobin-phospholipid emulsions (Table 8). These increases indicate that heat caused the myoglobin-phospholipid complex to be altered in some way. These increases seem to be in disagreement with the results at pH 7 (Table 6, page 50) where a significant decrease is seen with increasing temperature for percent myoglobin dispersed in the emulsion. If the myoglobin dispersions at pH 7.00, Table 6, are chosen for comparison,[8] it can be seen that for the emulsions heated to any temperature the percent myoglobin dispersed in an emulsion 3 M in phosphate (Table 8) is considerably less than that in the emulsion at pH 7.00. Therefore, it is possible to have a myoglobin phospholipid complex some of which precipitates at higher temperatures and some of which remains dispersed at higher temperatures. The part remaining dispersed is altered in some way by heating so that it is not precipitated in 3 M phosphate. Factors such as reaction sites on the myoglobin enter into this complex formation and alteration and will be discussed later in this chapter.

Significant differences also were observed between the percent myoglobin dispersed in solutions and in emulsions (Table 8). These

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[8] It is necessary to adjust pH of the dispersion to 7.00 before myoglobin can be isolated in 3 M phosphate, pH 6.6.

differences corresponded to those found in the dispersions at pH 7.00, Table 6, page 50, and have been discussed previously (page 49).

The data in Table 8, page 54, of myoglobin remaining dispersed in solutions and emulsions containing sarcoplasmic protein also were reported in Table 7, page 51. These data are included in Table 8 to show the comparison of percents of myoglobin remaining in both myoglobin and sarcoplasmic protein dispersions heated to 65 and 77°C.

The significant differences between percent myoglobin dispersed in myoglobin dispersions and in sarcoplasmic protein dispersions at 25 and 65°C (Table 8) are attributable to factors already discussed (page 53) concerning the damage to the myoglobin molecule during the blending process and concerning the myoglobin-phospholipid complex (page 53). At 65 and 77°C the percent myoglobin dispersed in the myoglobin solution was significantly larger than in the emulsion (Table 8). The observation that more myoglobin remains dispersed in heated myoglobin solutions than emulsions is in agreement with results shown in Table 6 where percent myoglobin dispersed in the heated solution was significantly larger than that in the corresponding myoglobin emulsion. At 77°C the observation that more myoglobin remains dispersed in heated myoglobin-containing solutions than in heated solutions containing sarcoplasmic proteins is in agreement with the results of several investigators who have found that myoglobin in pure solution is much more stable to heat precipitation than myoglobin in the presence of water-soluble proteins (Ledward et al., 1971) or in meat (Bernofsky et al., 1959 ; Siedler and Schweigert, 1959; Draudt, 1969).

Possible reaction sites and pH of the protein-containing solutions and protein-lipid emulsions. Means for acidic group equivalents of dispersed protein and of total protein are reported in Table 9. Heating beyond 56°C significantly increased the acidic group equivalents in dispersed protein of the solution, but no significant differences were found among 60, 65 and 77°C. No significant differences between temperatures for acidic group equivalents of dispersed protein in the emulsion were found. At 65 and 77°C significantly fewer acidic group equivalents were found in dispersed protein of the emulsion than that of the solution. Heating significantly increased the acidic group equivalents of total protein in protein-containing solutions between 25°C and the temperatures of 65 and 77°C, and heating significantly increased the acidic group equivalents of total protein in emulsion between 25°C and all other temperatures. Significant differences were not observed for the acidic group equivalents of total protein between the solution and the emulsion.

Hamm and Deatherage (1960) reported that there was no change in the acidic group equivalents of all muscle proteins between 20 and 40°C, but there was a decrease between 40 and 50°C, a marked decrease between 55 and 60°C and a continued decrease between 60 and 70°C. Changes in dye binding at the pH values used in their method (the same method used in the present investigation) would be due to establishment or breaking of covalent bonds such as peptide or ester bonds or strong complex bond and not to formation of salt cross linkages at pHs near the protein I.P. or changes in protein charge or in protein

TABLE 9

ACIDIC GROUP EQUIVALENTS OF DISPERSED AND TOTAL PROTEIN IN HEATED SOLUTIONS CONTAINING SARCOPLASMIC PROTEINS AND IN HEATED SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

Dispersion	Temperatures to Which Dispersions Were Heated				
	25°C	56°C	60°C	65°C	77°C
	Dispersed Protein				
	------Acidic group equivalents/10 kg protein--------				
Solution	3.7[b]	3.8[b]	5.0[a]	5.5[a]	6.5[a]
Emulsion	2.2[b]	4.1[b]	4.6[a,b]	3.3[b]	3.6[b]
	Total Protein				
	------Acidic group equivalents/10 kg protein--------				
Solution	3.7[b]	5.0[a,b]	5.8[a,b]	5.7[a]	6.6[a]
Emulsion	2.2[b]	5.4[a]	5.8[a]	4.9[a]	5.0[a]

[a,b]For dispersed protein or total protein values in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

structure which involved only a change in protein net charge would not influence dye-binding.

Denaturation of sarcoplasmic proteins decreased the solubility of myofibrillar proteins (Scopes, 1964). Bendall and Wismer-Pedersen (1962) reported that the fibrillar proteins of heat-coagulated tissues are probably covered by a layer of denatured sarcoplasmic proteins firmly bound to the surface of myofilaments.

The significant increases with heating that were found in the acidic equivalent groups of dispersed or total protein in protein-containing solutions (Table 9) indicate a possible breaking of covalent or strong complex bonds in the sarcoplasmic proteins which could provide suitable reaction sites for formation of covalent bonds between the sarcoplasmic proteins and other muscle constituents such as the myofibrillar proteins or lipids. At 65 and 77°C the significantly fewer acidic group equivalents of dispersed protein found in the protein lipid emulsion possibly indicates that bonds were formed between the reaction sites on the protein and the phospholipids.

The mean values for basic group equivalents of dispersed and total protein in heated sarcoplasmic protein dispersions are reported in Table 10. Significant differences were not observed between any of the values with increasing temperature or between solution and emulsion. These results agree with those of Hamm and Deatherage (1960), who found no significant change in the basic groups of muscle proteins during heating from 20 to 70°C.

TABLE 10

BASIC GROUP EQUIVALENTS OF DISPERSED AND TOTAL PROTEIN IN HEATED SOLUTIONS CONTAINING SARCOPLASMIC PROTEINS AND IN HEATED SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

Dispersion	Temperatures to Which Dispersions Were Heated				
	25°C	56°C	60°C	65°C	77°C
	Dispersed Protein[a]				
	------Basic group equivalents/10 kg protein---------				
Solution	13.9	14.1	14.3	14.3	13.6
Emulsion	13.7	15.2	15.4	14.9	15.1
	Total Protein[a]				
	------Basic group equivalents/10 kg protein---------				
Solution	13.9	12.8	14.0	14.2	13.4
Emulsion	13.7	14.3	14.1	13.9	14.0

[a]Significant differences were not observed at the $P < 0.05$ level.

The mean values for available lysine in dispersed protein and total protein of sarcoplasmic dispersions are reported in Table 11. Significant differences were not observed between values for any of the temperatures or between those for the solution and emulsion. This observation was anticipated since significant differences were not found for basic group equivalents. Available lysine is a measure of the lysine residue in which the ϵ-amino group is free (Kakade and Liener, 1969).

The pHs of the sarcoplasmic protein-containing solutions and emulsions are given in Table 12. Significant differences were not observed between any of the temperatures for the pH of the protein-containing solution but the pH of the solution did increase 0.14 of a pH unit from 25 to 77°C. The pH of the emulsion at 25°C was significantly lower than the pHs of the emulsions at 60, 65 and 77°C, and the pH of the emulsion at 56°C was significantly lower than that at 77°C.

The increases of pH in the emulsions were accompanied by increases in the acidic groups in the dispersed proteins in the emulsion. This increase in pH must be explained by other phenomena than those used by Hamm and Deatherage (1960) in explaining the increase in pH of cooked meat. It has been theorized that cleavage of salt-type cross linkages during heating could cause an increase in pH of solutions containing heat denatured proteins (Mirsky and Pauling, 1936).

Significantly lower pH values were found for the emulsion than for the solution. The lower pHs for the emulsion were accompanied by significantly higher values for acidic group equivalents of the

TABLE 11

AVAILABLE LYSINE RESIDUES OF DISPERSED AND TOTAL PROTEIN IN HEATED SOLUTIONS CONTAINING SARCOPLASMIC PROTEINS AND IN HEATED SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

Dispersion	Temperatures to Which Dispersions Were Heated				
	25°C	56°C	60°C	65°C	77°C
	Dispersed Protein[a]				
	-----Residues available lysine/10 kg protein--------				
Solution	8.1	9.9	8.9	9.4	9.4
Emulsion	6.6	9.0	7.6	8.1	8.0
	Total Protein[a]				
	-----Residues available lysine/10 kg protein--------				
Solution	8.1	8.8	6.8	7.1	6.3
Emulsion	6.7	7.4	7.2	6.4	6.6

[a]Significant differences were not observed at the $P < 0.05$ level.

TABLE 12

THE pH OF HEATED SOLUTIONS CONTAINING SARCOPLASMIC PROTEINS AND OF HEATED SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

Dispersion	Temperatures to Which Dispersions Were Heated				
	25°C	56°C	60°C	65°C	77°C
	pH				
Solution	5.55[d]	5.61[d]	5.62[d]	5.65[d]	5.69[d]
Emulsion	5.31[a]	5.39[a,b]	5.45[b,c]	5.49[b,c]	5.54[c]

[a,b,c,d]Values for pH in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

dispersed protein in the solution than in the emulsion at 65 and 77°C (Table 9, page 58).

The acidic group equivalents of heated myoglobin dispersions are shown in Table 13. Significant differences were not observed between any temperatures for acidic group equivalents of dispersed or total protein in the myoglobin solutions. In the myoglobin emulsion for dispersed protein the only significant difference was a decrease in acidic group equivalents between 25°C and the temperatures of 65 and 77°C. Significant differences in the acidic group equivalents of the total protein in the myoglobin emulsions were found among all temperatures. Heating the emulsion to higher temperatures generally caused a decrease in the acidic group equivalents of total protein in the emulsion, but heating from 65 to 77°C caused a significant increase. The dispersed protein in the emulsion at 25°C and the total protein in the emulsion at 25 and 77°C had significantly more acidic group equivalents than the respective protein had in solution at the same temperatures.

The range of the acidic group equivalents (15-22) per mole of myoglobin, dispersed or total, found in the myoglobin solution is below the number of total acidic residues (32/mole myoglobin) found by Atassi et al. (1970) who determined the amino acid composition of bovine myoglobin. The acidic group equivalents in myoglobin are the carboxyl groups of aspartic and glutamic acid and the hydroxyl group of tyrosine (Paul, 1972b).

The number of acidic group equivalents (54.4/mole myoglobin) found in myoglobin emulsions at 25°C (Table 13) is much greater than

TABLE 13

ACIDIC GROUP EQUIVALENTS OF DISPERSED PROTEIN AND TOTAL PROTEIN IN HEATED MYOGLOBIN-CONTAINING SOLUTIONS AND IN HEATED MYOGLOBIN-PHOSPHOLIPID EMULSIONS

Dispersion[d]	Temperatures[d] to Which Dispersions Were Heated		
	25°C	65°C	77°C
	Dispersed Protein		
	---Acidic group equivalents/mole myoglobin----		
Solution	22.0[a]	19.2[a]	15.0[a]
Emulsion	54.4[b]	20.8[a]	25.0[a]
	Total Protein		
	---Acidic group equivalents/mole myoglobin----		
Solution	22.0[a]	19.2[a]	15.0[a]
Emulsion	54.4[b]	27.7[a]	33.1[c]

[a,b,c]Means for dispersed protein or total protein in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

[d]The temperature-dispersion interaction for dispersed protein is significant at the $P < 0.05$ level.

the total acidic group residues/mole myoglobin (32) found by Atassi et al. (1970). The acidic group equivalents in the present investigation were determined by the safranine-dye binding method of Hamm and Deatherage (1960) and possibly some interaction between the phospholipids and myoglobin caused increased binding of safranine resulting in the abnormally high value for the acidic group equivalents in myoglobin. Heating the myoglobin emulsion caused a drastic reduction in the number of acidic group equivalents per mole myoglobin and these values at 65 and 77°C in the emulsion are either below or close to total acidic group residues per mole bovine myoglobin reported by Atassi et al. (1970).

Basic group equivalents of dispersed protein and total protein in heated myoglobin dispersions are reported in Table 14. At 25°C dispersed or total protein in a protein-containing solution had significantly more basic group equivalents than the dispersed or total protein had in the emulsion. The values of the basic group equivalents/mole myoglobin in the myoglobin-containing solutions at all temperatures are higher (37.5-40.9) than the basic group residues per mole of bovine myoglobin (33) reported by Atassi et al. (1970). The basic groups are the lysine, arginine and histidine side chains in the protein (Paul, 1972b).

The difference in the basic group equivalents between the solution and the emulsion at 25°C (Table 14) suggests that phospholipids are bound to basic groups of myoglobin in the emulsion. However, because the value of basic group equivalents per mole of myoglobin is

TABLE 14

BASIC GROUP EQUIVALENTS OF DISPERSED AND TOTAL PROTEIN IN HEATED MYOGLOBIN-CONTAINING SOLUTIONS AND IN HEATED MYOGLOBIN-PHOSPHOLIPID EMULSIONS

Dispersion	Temperatures to Which Dispersions Were Heated		
	25°C	65°C	77°C
	Dispersed Protein		
	----Basic group equivalents/mole myoglobin-------		
Solution	40.9[a]	37.5[a,b]	39.6[a,b]
Emulsion	29.6[b]	35.3[b]	37.2[b]
	Total Protein		
	----Basic group equivalents/mole myoglobin-------		
Solution	40.9[a]	37.5[a,b]	39.6[a,b]
Emulsion	29.6[b]	30.7[b]	31.6[b]

[a,b]Means for dispersed protein or total protein in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

greater than the reported value of basic group residues per mole of myoglobin (Atassi et al., 1970), this observation cannot be a definite conclusion just from the data in Table 14.

Available lysine of dispersed protein and total protein in heated myoglobin dispersions is shown in Table 15. The only significant difference found was between the solution and emulsion for the available lysine in total protein at 25°C. The myoglobin in the emulsion had significantly less available lysine than that in the myoglobin-containing solution.

The value for available lysine residues per mole of myoglobin contained in the solution at 25°C (20.1) is fairly close to the reported value (19) of total residues of lysine in a mole of bovine myoglobin (Atassi et al., 1970). The difference could be attributed to experimental error in the procedure for determination of available lysine (Kakade and Liener, 1969). The significant difference between solution and emulsion for the available lysine at 25°C (Table 15) shows that phospholipids in the emulsion are bound to the ϵ-NH_2 group of lysine residues in the myoglobin molecule. It also shows that the significant difference observed in Table 14 is probably a real difference since lysine residues make up 57 percent of the total basic group equivalents in myoglobin.

The temperature-dispersion interaction seen in Table 15 reflects the tendency for available lysine in a myoglobin solution to decrease and that in the emulsion to increase with increasing temperature. These results suggest that heating decreased available lysine (caused new

TABLE 15

AVAILABLE LYSINE RESIDUES OF DISPERSED AND TOTAL PROTEIN IN HEATED MYOGLOBIN-CONTAINING SOLUTIONS AND IN HEATED MYOGLOBIN-PHOSPHOLIPID EMULSIONS

Dispersion[c]	Temperatures[c] to Which Dispersions Were Heated		
	25°C	65°C	77°C
	Dispersed Protein		
	----Residues available lysine/mole myoglobin-----		
Solution	20.1[a]	19.7[a]	11.8[a]
Emulsion	11.7[a]	17.2[a]	19.5[a]
	Total Protein		
	----Residues available lysine/mole myoglobin-----		
Solution	20.1[a]	19.7[a,b]	11.8[a,b]
Emulsion	11.7[b]	15.1[b]	16.3[b]

[a,b]Means for dispersed protein or total protein in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

[c]The temperature-dispersion interaction for dispersed protein and total protein is significant at the $P < 0.01$ level.

bonds to form at the ϵ-NH_2 group) in myoglobin and broke bonds formed between phospholipids and myoglobin at the ϵ-NH_2 groups of lysine in the emulsion.

In the myoglobin-phospholipid emulsion of the present investigation phospholipids (0.8000 g/100 g emulsion) were in excess of the myoglobin (0.2000 g/100 g emulsion). Gulik-Krzywicki et al. (1969) investigated a phospholipid interaction with a water soluble protein in aqueous dispersion in which the amount of lipid was in excess of the amount of protein. They showed by X-ray diffraction of the phases formed that there were contacts between non-polar parts of the lipids and non-polar regions of the protein molecules as well as between the polar regions. Changes in the spectra at 190-240 nm, the region of peptidic absorption, were reported which indicated that the conformation of the native protein molecule was somewhat disturbed by this interaction. It is possible that these reported interactions and phenomena observed by Gulik-Krzywicki et al. (1969) were present in the myoglobin phospholipid emulsions and were in some ways responsible for the phenomena observed in the present investigation.

The pHs of heated myoglobin-containing solutions and of heated myoglobin emulsions are shown in Table 16. The pHs of the emulsions were significantly lower than those of the solutions for all temperatures. The pH values in the emulsion increased significantly when the emulsion was heated from 25°C to higher temperatures. The pH tended to increase with increasing temperature of the solution, but differences were not significant.

TABLE 16

THE MEASURED pH OF HEATED MYOGLOBIN-CONTAINING SOLUTIONS AND HEATED MYOGLOBIN-PHOSPHOLIPID EMULSIONS

Dispersion	Temperatures to Which Dispersions Were Heated		
	25°C	65°C	77°C
	----------pH----------		
Solution	5.47[a]	5.65[a]	5.71[a]
Emulsion	4.07[b]	4.34[c]	4.40[c]

[a,b,c]Values for pH in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

The significant difference in pH between the solution and the emulsion at 25°C probably is attributable to the significant difference in acidic group equivalents of dispersed protein (Table 13, page 65) and in basic group equivalents of dispersed protein (Table 14, page 67) between the solution and the emulsion (Hamm and Deatherage, 1960).

The significant increase in pH in the heated emulsions was most likely because of the significant decrease in acidic group equivalents of dispersed protein in heated emulsion as compared with the acidic group equivalents of dispersed protein at 25°C (Table 13). The significant difference in pH between solution and emulsion at 65 and 77°C cannot be explained by significant increases or decreases in acidic group equivalents.

Electrophoretic pattern of bovine sarcoplasmic proteins and bovine myoglobin. Densitometric tracings of the electrophoretic patterns of bovine sarcoplasmic proteins in a protein-containing solution at 25, 56, 60 and 65°C are shown in Figure 8, and in Figure 9 are the corresponding electrophoretic patterns. For any one temperature the numbered bands in Figure 9 correspond to the respective numbered peaks in Figure 8. The electrophoretic pattern of sarcoplasmic proteins at 25°C in Figure 8 represents an extract from unheated meat. This pattern has a smaller number of peaks (11 versus 17) than a corresponding electrophoretogram shown by Laakkonen et al. (1970). The difference in the number of peaks probably is because: (1) some proteins which precipitated during dialysis of the sarcoplasmic protein extract were discarded;

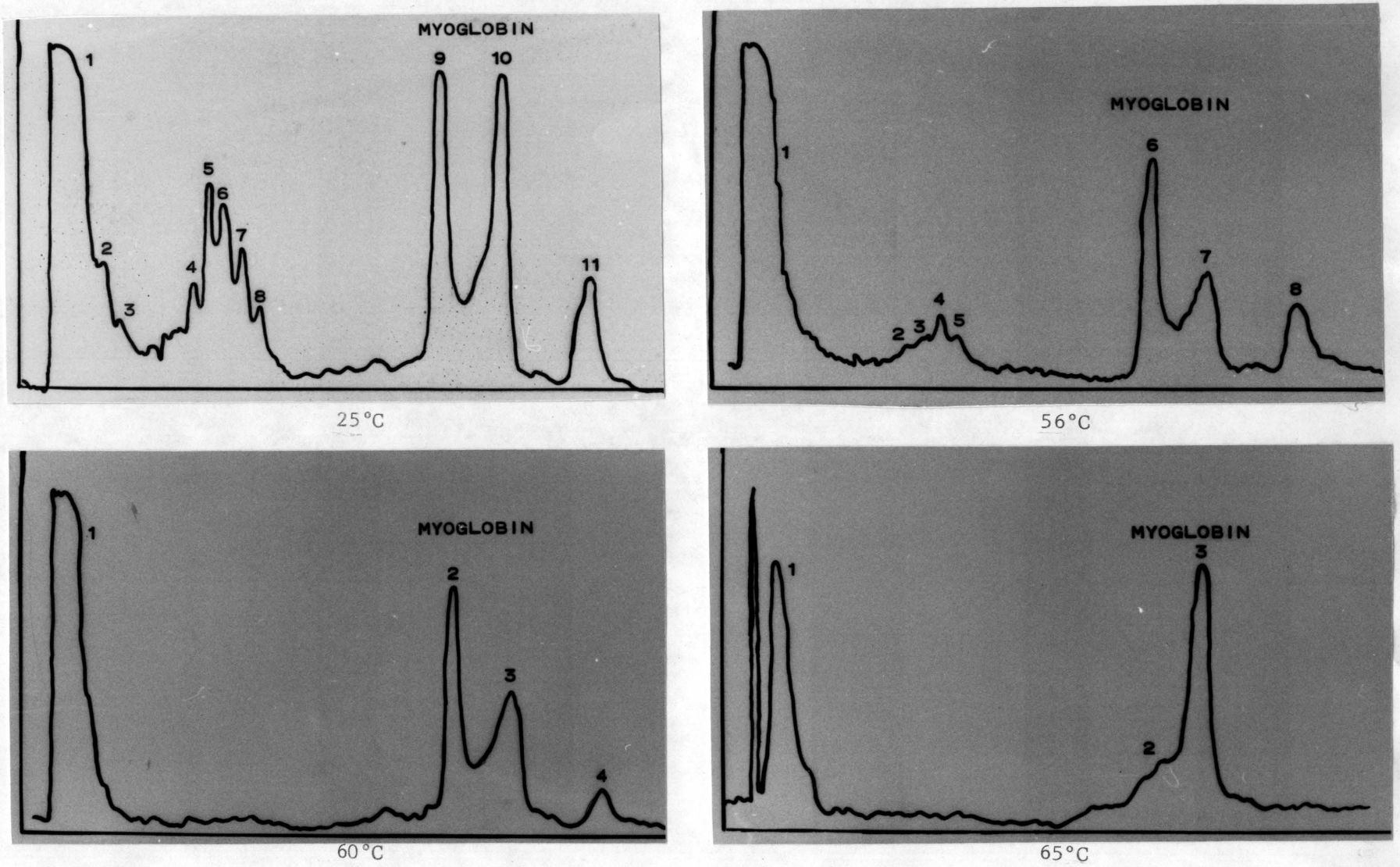
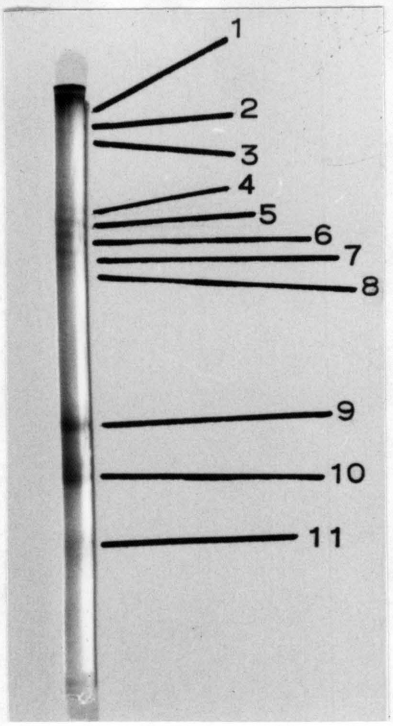
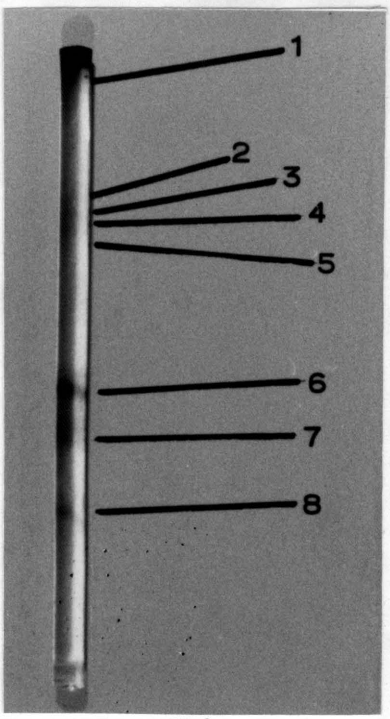


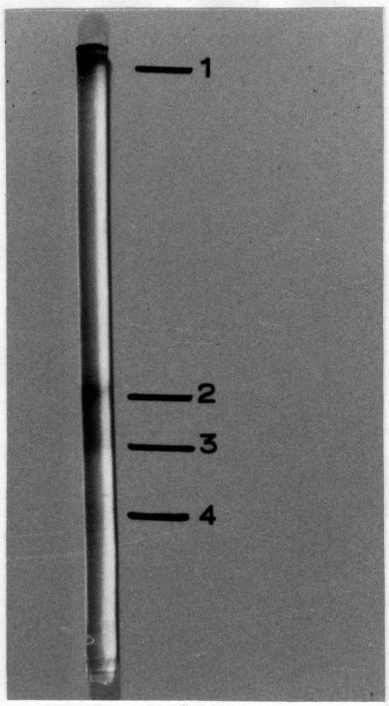
Figure 8. Densitometric tracings of the electrophoretic patterns of bovine sarcoplasmic proteins in solutions at 25, 56, 60 and 65°C.



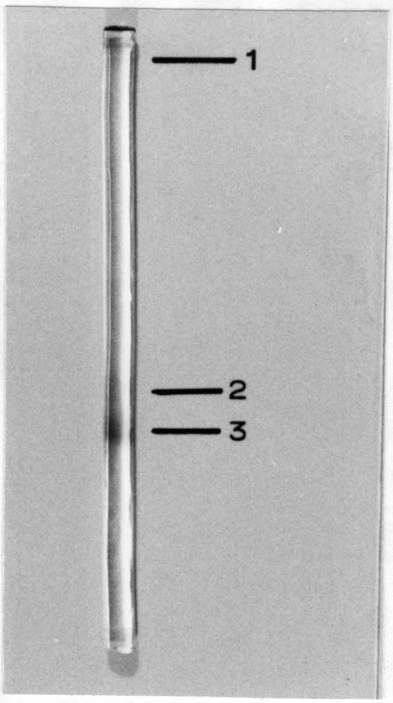
25°C



56°C



60°C



65°C

Figure 9. Electrophoretic patterns of bovine sarcoplasmic proteins in solutions at 25, 56, 60 and 65°C.

(2) some of the minor bands at 25°C in Figure 8 are not numbered; and (3) the proteins in the solutions at 25°C have been subjected to mechanical stress which could have altered their molecular conformation so that they were precipitated at the pH of the electrophoretic run (8.1). The peaks 9 and 10 in the tracing at 25°C in Figure 8 correspond to bands 7 and 8 in the electrophoretograms of Laakkonen et al. (1970). Bands 7 and 8 were the most intensely colored myoglobin bands found by Laakkonen et al. (1970). The relative sizes of the myoglobin peaks, 9 and 10, do not correspond to the normal pattern found for bovine myoglobin in polyacrylamide disc gel electrophoresis (Höyem and Thorson, 1970; Laakkonen et al., 1970). In the reports of the preceding investigators, the slower moving band of myoglobin was approximately one-fourth to one-third the size of the faster moving myoglobin band, represented by peak 10 and 25°C in Figure 8, page 73. The most likely difference in the extract in the present investigation that could have caused the alteration of myoglobin electrophoretic pattern would have been the blending of the protein solutions for 30 seconds in a microblendor. In sarcoplasmic protein solutions that had not been blended a normal myoglobin electrophoretic pattern was obtained (Figure 2, page 16).

Bands 4-8 were the first proteins to be affected by heating. At 56°C the number of discernible bands in this area of electrophoretic pattern had decreased to 4 (Figure 8) and the peak areas were much reduced. By 60°C the bands numbered 4-8 in the 25°C tracing had disappeared completely. Laakkonen et al. (1970) reported that the

bands just preceding the myoglobin band in water extracts of meat or from meat drip were the most heat sensitive. Scopes (1970) listed glycolytic and associated enzymes such as phosphorylase b, phosphorylase isomerase, phosphofructo kinase, enolase, etc. as being unstable at 55°C, pH 5.5. These protein bands which disappear by 60°C are probably such enzymes.

Laakkonen et al. (1970) identified the bands appearing after the myoglobin bands in their electrophoretograms as myoalbumin. They found that some of the bands disappeared when meat was heated to 57°C but there still were myoalbumin bands in extracts of meat heated to 60°C. Peak 11 (25°C), peak 8 (56°C) and peak 4 (60°C) in Figure 8, page 73, represent the only myoalbumin band found in the present investigation. The peak area decreased with heating until it was absent by the temperature of 65°C.

The area in peak 1 (which is probably more than one protein) Figure 8 decreases by 60°C and decreases still more by 65°C. Laakkonen et al. (1970) found that the slowest moving bands in water extracts of cooked meat or in the drip from the meat were affected by heating to 60°C. The slowest moving bands in their investigation are represented by peak 1 in Figure 8.

The area of the myoglobin peak at 56°C appears to be smaller than that of the myoglobin peak at 25°C; however, significant differences in myoglobin concentration were not found between 25 and 56°C in the heated sarcoplasmic protein solution (Table 7, page 51).

The change in the electrophoretic pattern of myoglobin with increasing temperature must be caused by heat-induced conformational changes in the myoglobin molecule itself. Bodwell and McClain (1971) reported that although electrophoretically different myoglobins have been isolated, the differences appear to be caused by shifts in the location of amide groups in the polypeptide chains. It is not unreasonable, therefore, to expect changes in the electrophoretic pattern of myoglobin during heating.

The decrease or absence of various sarcoplasmic protein bands shown by the tracings of the electrophoretic pattern in Figure 8, page 73, at any one temperature corresponds to significant decreases in the percent protein remaining dispersed with each temperature increment at pH 7.00 (Table 5, page 47).

The densitometric tracings of the electrophoretic patterns of bovine sarcoplasmic proteins in emulsions at 25°C and protein emulsions heated to 56, 60 and 65°C are shown in Figure 10, and the corresponding electrophoretic patterns are shown in Figure 11. The electrophoretic pattern of myoglobin in the emulsions was found to be different from the electrophoretic pattern of the myoglobin in the solution of sarcoplasmic proteins (Figure 9, page 74). The pattern of myoglobin bands in the emulsion more closely resembles the electrophoretic pattern of bovine myoglobin in polyacrylamide gels reported by Höyem and Thorson (1970) and by Laakkonen et al. (1970). The observations that were made about the disappearance of protein peaks in heated solutions of sarcoplasmic proteins also can be made

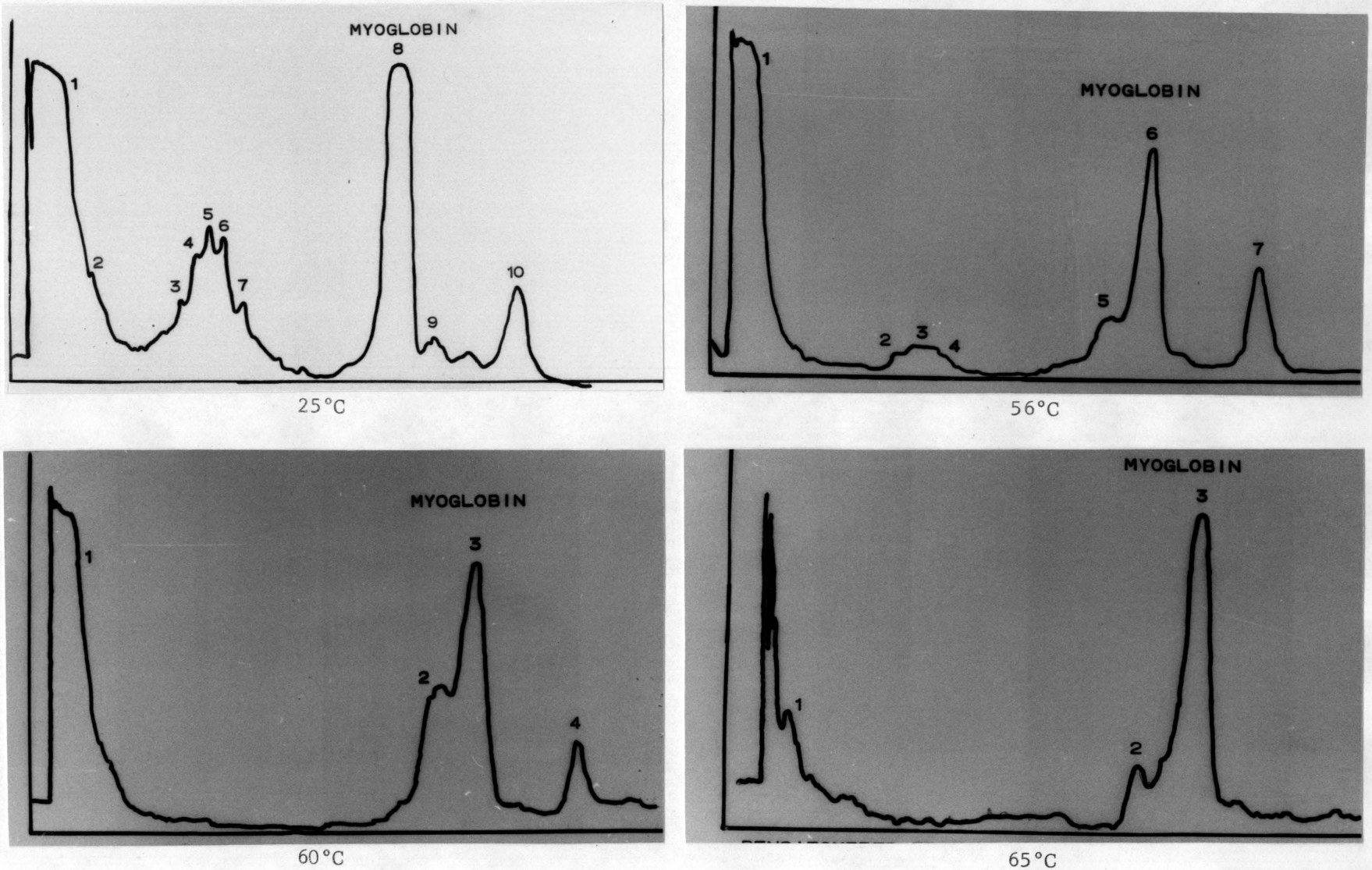
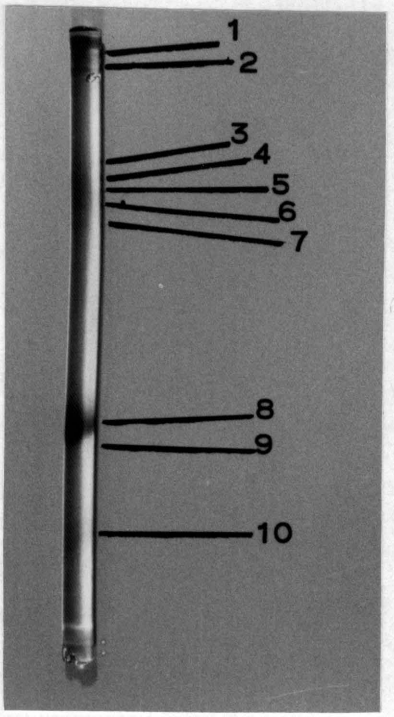
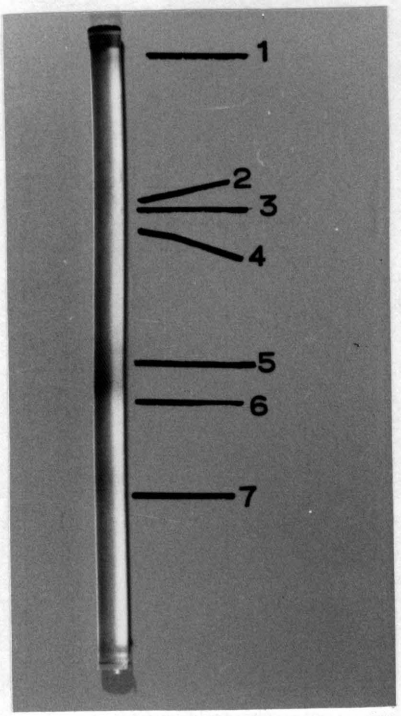


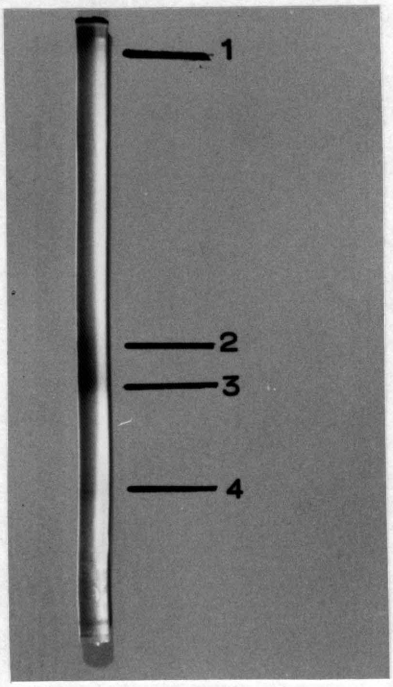
Figure 10. Densitometric tracings of the electrophoretic patterns of bovine sarcoplasmic proteins in protein-phospholipid emulsions at 25°C and after heating to 56, 60 and 65°C.



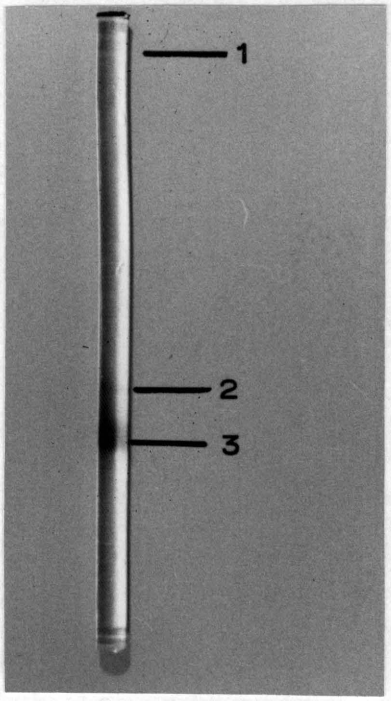
25°C



56°C



60°C



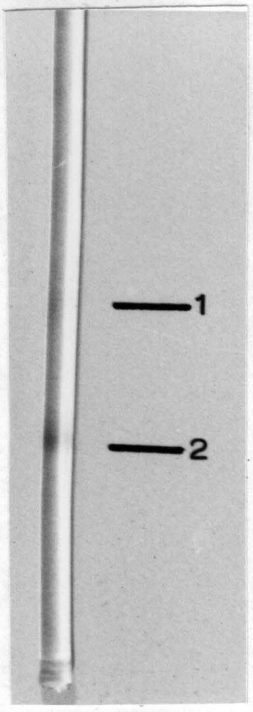
65°C

Figure 11. Electrophoretic patterns of bovine sarcoplasmic proteins in protein-phospholipid emulsions at 25°C and heated to 56, 60 and 65°C.

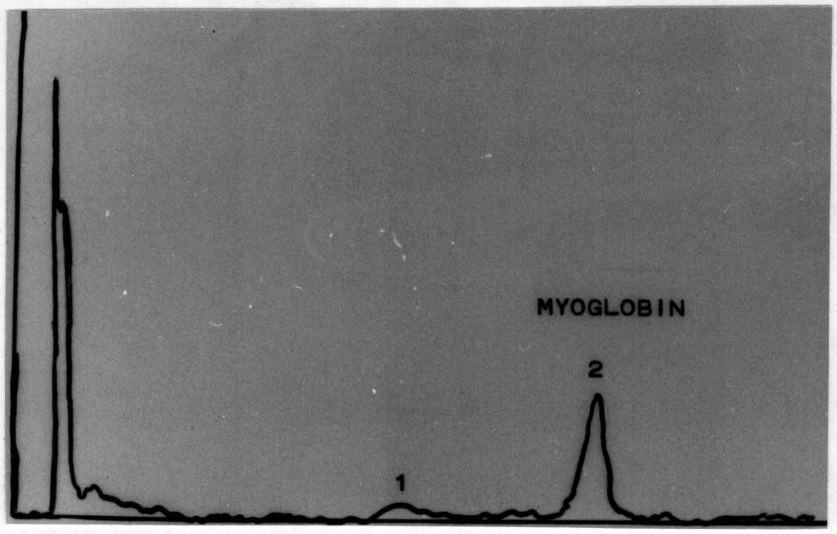
about the disappearance of the same protein peaks in heated emulsions of sarcoplasmic protein-phospholipids. If peaks 3-7 in the 25°C tracing in Figure 10 are compared with the same peaks, numbered 4-8, in Figure 8, page 73 (25°C), it can be seen that there appears to be a larger area under the peaks in Figure 8 than under the peaks in Figure 10. This difference probably reflects the significant difference that was found in the percent protein remaining dispersed between solution and emulsion of sarcoplasmic proteins at 25°C (Table 5, page 47).

The electrophoretic patterns and the densitometric tracing of the patterns of bovine sarcoplasmic proteins in solution and in protein-phospholipid emulsions heated to 77°C are shown in Figure 12. The first significant decrease found in myoglobin concentration in heated solutions of bovine sarcoplasmic proteins was between 65 and 77°C (Table 7, page 51), and this is shown dramatically by comparison of the tracing for 65°C in Figure 8 with the tracing of sarcoplasmic proteins in solution at 77°C in Figure 12. A comparison of the two tracings in Figure 12 shows that the area under the myoglobin peak in the emulsion appears to be larger than the area under the myoglobin peak in the solution. There was a significant difference between the percent myoglobin dispersed in the emulsion (45.3 percent) and in the solution (39.8 percent) in the sarcoplasmic protein dispersions at pH 7.00 (Table 7).

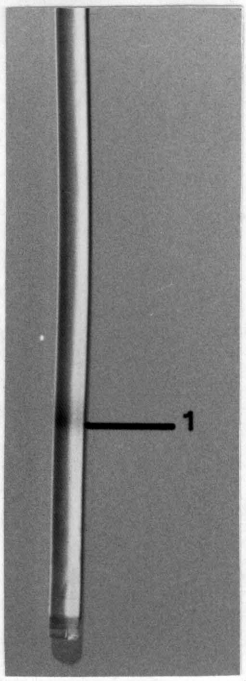
The electrophoretic patterns of bovine myoglobin dispersed in solution at 25°C and in heated solutions at 65 and 77°C and the



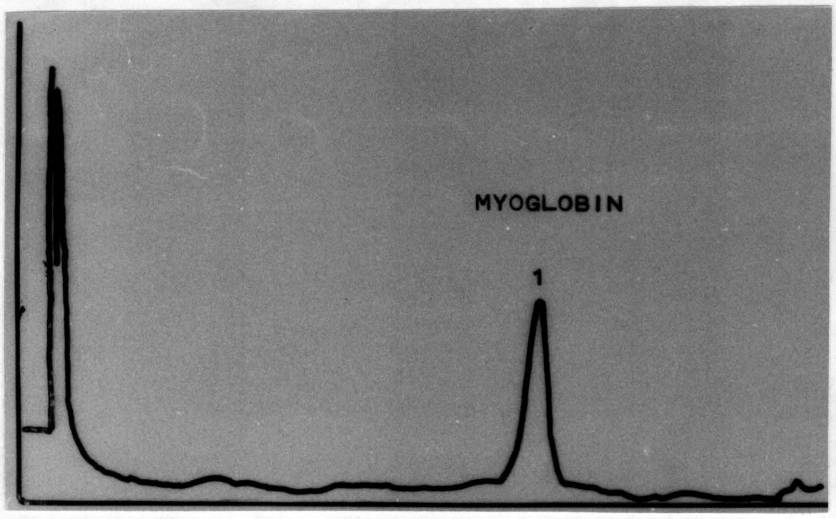
Electrophoretic pattern, solution heated to 77°C



Densitometric tracing, solution heated to 77°C



Electrophoretic pattern, emulsion heated to 77°C

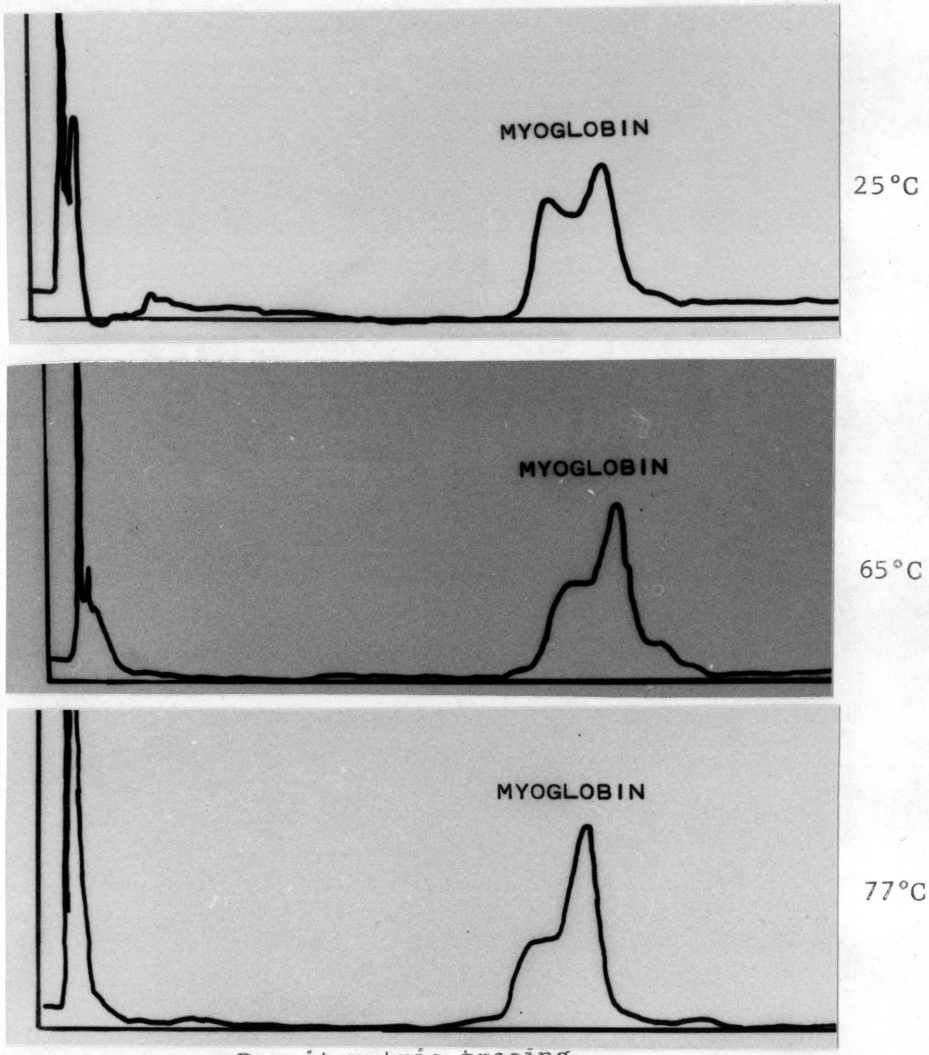


Densitometric tracing, emulsion heated to 77°C

Figure 12. Electrophoretic patterns of bovine sarcoplasmic proteins in solutions and in protein-phospholipid emulsions heated to 77°C and densitometric tracings of the patterns.

densitometric tracings of those patterns are shown in Figure 13. The peaks of purified myoglobin in the densitometric tracings for 25, 65 and 77°C (Figure 13) correspond to the more intensely colored bands of myoglobin (7 and 8) found in water-soluble bovine proteins by Laakkonen et al. (1970). The areas under the two myoglobin peaks in the 25°C tracing are not proportioned like the area of band 8 in relation to band 7 (a normal myoglobin pattern, Laakkonen et al., 1970). The isolation procedure for preparation of purified bovine myoglobin or the blending of the solution could have caused this variation in myoglobin pattern. Heating the solution containing myoglobin caused a more normal myoglobin pattern in the tracings at 65 and 77°C (Figure 13). Significant differences were not found in percent myoglobin dispersed in heated solutions containing myoglobin (pH 7.00, Table 6, page 50) and there seem to be only small decreases in the area in the first myoglobin band in the tracings in Figure 13 with increasing temperature from 25 to 65 and 77°C.

The electrophoretic patterns of myoglobin in myoglobin-phospholipid emulsions at 25, 65 and 77°C and the densitometric tracings of those patterns are shown in Figure 14. These data for 25°C show that little myoglobin migrated into the gel; in the emulsion heated to 65°C more of the myoglobin migrated into the gel; and the myoglobin peaks in the emulsion heated to 77°C were the approximate size and shape of the myoglobin peaks of the solution heated to 77°C (Figure 13).



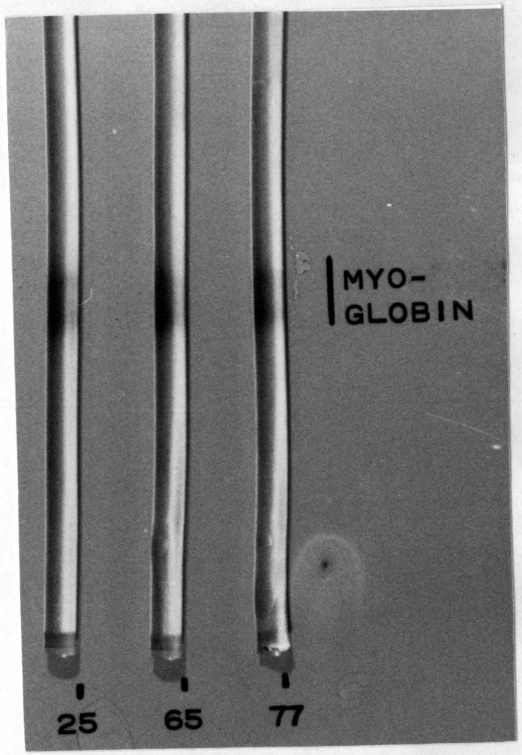
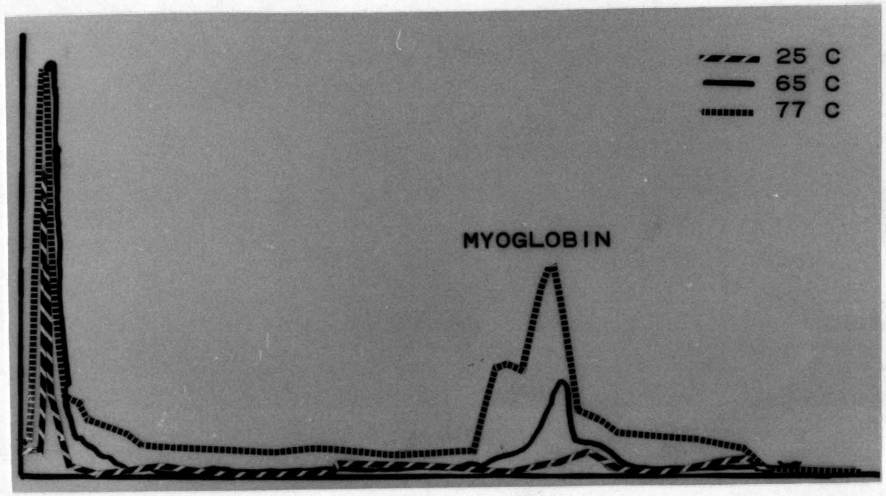
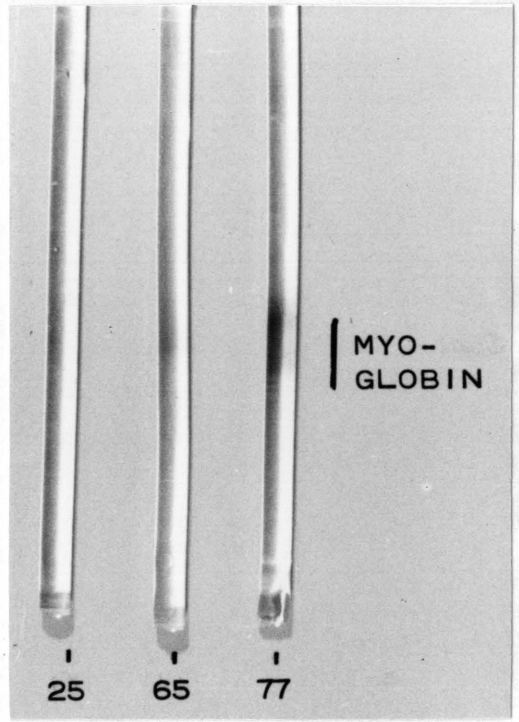


Figure 13. Electrophoretic patterns of myoglobin dispersed in solutions at 25, 65 and 77°C and densitometric tracings of the patterns.



Densitometric tracings



Electrophoretic patterns

Figure 14. Electrophoretic patterns of myoglobin in myoglobin-phospholipid emulsions at 25, 65 and 77°C and the densitometric tracings of the patterns.

Summary

Bovine sarcoplasmic protein solutions (1.4 g protein/100 g solution) and sarcoplasmic protein-phospholipid emulsions (1.4 g protein and 0.8 g phospholipids/100 g emulsion) were heated from 25°C to end point temperatures of 56, 60, 65 and 77°C at a rate of temperature increase of 1°C/min. Bovine myoglobin solutions (0.2 g protein/100 g solution) and bovine myoglobin-phospholipid emulsions (0.2 g protein and 0.8 g phospholipids/100 g emulsion) were heated from 25°C to end point temperatures of 65 and 77°C at the same rate of temperature increase. The following measurements were made on dispersions at 25°C and on all heated dispersions: (1) pH; (2) percent protein remaining dispersed at the pH of the dispersion and after pH of the dispersion that had been heated was adjusted to 7.00; (3) myoglobin concentration after isolation in 3 M phosphate solution; (4) available lysine, basic group equivalents and acidic group equivalents of dispersed and total protein in the dispersions; and (5) densitometric tracings of the polyacrylamide disc gel electrophoretic patterns of dispersed proteins.

Significant decreases ($P < 0.05$) in percent dispersed protein in solutions containing sarcoplasmic proteins were found with increasing end point temperatures up to 77°C; a significant decrease in percent dispersed protein in emulsions containing sarcoplasmic protein resulted from a temperature increase from 25 to 56°C but no decrease occurred with further heating. After the pH of unheated and heated dispersions was adjusted to 7.00 the emulsions contained significantly

less dispersed protein ($P < 0.05$) than the protein-containing solutions. The formation of sarcoplasmic protein-phospholipid complexes that were more stable to heat precipitation at the pH range of 5.30 to 5.70 but less stable to higher pH (7.00) than the heated sarcoplasmic proteins alone is indicated.

Heating both of the sarcoplasmic protein dispersions from 25 to 56°C, from 56 to 65°C and from 65 to 77°C caused significant decreases in dispersed total heme pigment with each temperature increase and the emulsion had significantly less dispersed total heme protein at 56, 60 and 65°C than the protein-containing solution. It was necessary to heat the protein-containing solution from 65 to 77°C and the emulsion from 60 to 65°C before significant decreases were found in the myoglobin concentrations of the dispersions. The emulsion heated to 65°C contained significantly less dispersed myoglobin and the emulsion heated to 77°C contained significantly more dispersed myoglobin than the corresponding protein-containing solutions. All significant differences were at the $P < 0.05$ level. The formation of a myoglobin-protein-phospholipid complex that was less table to heat precipitation than a myoglobin-protein complex at 65°C but more stable to heat precipitation at higher temperatures (77°C) is indicated.

Sarcoplasmic protein-phospholipid emulsions had significantly lower pHs than the solutions containing sarcoplasmic proteins, but significant differences were not observed between solution and emulsion for available lysine, or basic group equivalents of total or dispersed protein or for acidic group equivalents of total protein at any

temperature. Significant differences in any of these measurements would have indicated the possible formation or breaking of covalent bonds such as peptide or ester bonds or strong complex bonds. Interactions between phospholipids and proteins did occur, and most of these interactions probably were weak polar bonds.

Heating sarcoplasmic protein dispersions to 77°C did cause significant increases ($P < 0.05$) in acidic group equivalents of dispersed and total protein in protein-containing solutions and of total protein in the emulsion. The protein-containing solutions also had significantly greater acidic group equivalents at 65 and 77°C than the emulsions. This suggested that heating broke covalent or strong complex bonds involving acidic groups of protein in the protein-containing solutions. These broken bonds could provide suitable reaction sites for interaction with other proteins or muscle constituents.

Heating to increasing end point temperatures generally caused significant increases in the pH of emulsions containing sarcoplasmic proteins and these increases apparently involved changes in weak polar bonds. Heating did not cause significant differences in basic group equivalents or available lysine of total or dispersed protein in sarcoplasmic protein-containing solutions or emulsions.

The densitometric tracings of the electrophoretic pattern of bovine sarcoplasmic proteins in dispersions containing the proteins (solution and emulsion) at 25°C and at each end point temperature showed that: (1) the electrophoretic pattern of myoglobin was altered by mechanical blending of protein-containing solutions but not by blending

of the emulsions; (2) heating both dispersions to 60°C caused the protein peaks which probably represented glycolytic enzymes to disappear completely; (3) heating both dispersions to 65°C caused the protein peak which probably represented a myoalbumin to disappear; (4) myoglobin peaks in the emulsion and in the protein-containing solution were still present in dispersions heated to 77°C, but the peaks were smaller than at lower temperatures; and (5) the myoglobin peak in the emulsion heated to 77°C appeared to be larger than the myoglobin peak in the protein-containing solution heated to 77°C.

Heating the myoglobin solution caused no change at all in percent dispersed protein and heating the myoglobin-phospholipid emulsion caused no significant decreases in percent dispersed myoglobin. Heated emulsions at the measured pH and after pH adjustment contained significantly less dispersed myoglobin than the corresponding heated solutions. The formation of a myoglobin-phospholipid complex that was less stable to heat precipitation and to a higher pH (7.00) than myoglobin is indicated.

Many other interesting phenomena also were observed in the myoglobin-phospholipid emulsions. At 25°C there apparently were interactions between phospholipids and myoglobin that caused: (1) increased dye binding at pH 11.5 (method of Hamm and Deatherage, 1960) resulting in abnormally high values for acidic group equivalents per mole of myoglobin; (2) significantly lower pHs in the myoglobin-containing emulsion than in the myoglobin-containing solution ($P < 0.05$); (3) precipitation of myoglobin in 3 M phosphate solutions, pH 6.6; and (4) failure

of myoglobin to migrate in polyacrylamide disc gel electrophoresis at a pH of 8.1. Heating the myoglobin-phospholipid emulsions from 25 to 65 and 77°C caused: (1) the acidic group equivalents of myoglobin to return below the total number of acidic group residues per mole myoglobin; (2) the pH of the emulsion to increase significantly ($P < 0.05$); (3) the solubility of myoglobin in 3 M phosphate solution to increase significantly ($P < 0.05$); (4) the amount of myoglobin migrating in gels at pH 8.1 to increase greatly; and (5) the number of ϵ-NH_2 groups of lysine in the myoglobin to increase significantly ($P < 0.05$). Interactions between myoglobin and phospholipids might involve weak polar and nonpolar bonds and did involve either covalent bonding or strong complex bonding between the basic groups of myoglobin, especially the ϵ-NH_2 of lysine, and the phospholipids.

CHAPTER IV

PROTEIN-PHOSPHOLIPID INTERACTIONS IN HEATED PHOSPHOLIPID EMULSIONS CONTAINING BOVINE MYOGLOBIN OR SARCOPLASMIC PROTEINS. II. PHOSPHOLIPID CONTENT AND CLASSES ASSOCIATED WITH PROTEIN

Introduction

Several investigators have shown that myoglobin derivatives precipitate in meat (at temperatures of 60°C or above) about 15-30°C below the temperatures required in pure solution at the same pH (Bernofsky et al., 1959; Siedler and Schweigert, 1959; Draudt, 1969). In aqueous muscle extracts the precipitation of myoglobin at 60-65°C is caused by higher molecular weight proteins (Ledward, 1971). These investigators who studied the thermal denaturation of myoglobin in meat or in aqueous extracts did not study myoglobin interactions with non-protein constituents such as lipids.

The effect of heat on phospholipids in some foods has been reported. Macy and coworkers (1964) heated amino nitrogen constituents in lyophilized diffusates from aqueous extracts of beef, pork and lamb for one hour in a boiling water bath and found that heating caused marked increases of phosphoethanolamine in samples from all three animal species. Hydrolysis of phospholipids during the heating of meat was indicated. Nakanishi and Suyama (1967) heated phospholipids isolated from bovine thigh meat and found that heating caused a marked

decrease in phosphatidyl ethanolamine and phosphatidyl serine and an increase in the contents of phosphatidic acid and water-soluble components containing phosphorus. Campbell and Turkki (1967) found that drip phospholipids from cooked beef consisted largely of cephalins (phosphatidyl ethanolamine) and that the cooked meat contained a significantly lower cephalin concentration than the raw meat. Nakanishi and Kaya (1970a,b) reported that heating raw milk for 30 minutes at 63 and 93°C decreased phospholipid phosphorus content, respectively, to 96 and 86.5 percent of initial value and that the decomposition of cephalin was greater than that of either lecithin (phosphatidyl choline) or sphingomyelin. A greater rate of decomposition of phospholipids heated in milk than of phospholipid heated in water also was shown by Nakanishi and Kaya (1970b).

Interactions between lipids and proteins have been intensively studied in wheat flour dough and in biological systems (Fullington, 1969). Generally interactions between phospholipids and proteins are polar in nature and lipids are bound to the protein (Fullington, 1969; Barratt et al., 1968; Gulik-Krzywicki et al., 1969). Indications of polar interactions were observed between phospholipids and myoglobin in this present investigation (page 70).

Much study of lipid binding by wheat flour proteins has used solvent extractability for determining bound lipid (Fullington, 1969). Chiu and Pomeranz (1966) in an investigation of changes in extractability of lipids during bread making used petroleum ether to extract free lipid and a more polar solvent system, chloroform-methanol, to

extract bound lipid. Other investigators have used polar solvents such as water saturated butanol to extract bound lipids from wheat flour proteins during bread making (Hoseney et al., 1970). The free lipid that is extracted by petroleum ether included unbound lipids and lipids held by hydrophobic bonding (Hoseney et al., 1970).

Phospholipid classes, lecithin and cephalin, were shown to interact with a variety of proteins such as cytochrome c (Shipley et al., 1969; Barratt et al., 1968), soluble soybean protein (Rzhekhin and Krasilnov, 1965), wheat flour dough proteins (Fullington, 1969), etc. Cephalins were reported to show a higher affinity than lecithins for soluble soybean protein (Rzhekhin and Krasilnov, 1965).

This study was initiated: (1) to determine effect of heating on bovine phospholipids in an aqueous emulsion and in protein-phospholipid emulsions and (2) to determine phospholipid content, bound and free, and phospholipid classes associated with protein in heated bovine myoglobin- and bovine sarcoplasmic protein-phospholipid emulsions. Such information should add to the knowledge of the degradation of phospholipids and denaturation of myoglobin during the cooking of meat.

Materials and Methods

Purified bovine myoglobin, water extracts of bovine sarcoplasmic proteins, and phospholipids were prepared as described on pages 11, 13 and 37, respectively.

Preparation, heating and evaluation of heated phospholipid emulsions. Phospholipids that were dried as described on page 38 were

blended with cold distilled water (0.8 g phospholipids/100 g water) for 30 seconds in a micro jar on a Waring blendor at a setting of 90 on a Powerstat variable transformer. Enough of the phospholipid emulsion was prepared at one time to allow a sample of the emulsions to be evaluated at each sampling temperature. The emulsion was heated from 25 to 77°C under a nitrogen atmosphere at a rate of temperature increase of 1°C/min. A sample of approximately 20 g was removed from the emulsion by pipet at 25°C and at end point temperatures of 56, 60, 65 and 77°C. Two emulsions were prepared, heated and sampled in this way. All containers and pipets used in these procedures were rinsed with small portions of chloroform, and the rinse added to a preweighed 250 ml beaker for recovery of phospholipids.

Immediately after preparation the emulsion was placed in a 250 ml round bottom flask with a thermometer well. The flask was suspended in a water bath (Figure 5, page 40) which had been set to increase the temperature at the desired rate. A thermocouple was inserted in the flask and the temperature was monitored by a Honeywell temperature recorder during heating. At 25°C (the temperature of bath and emulsion before actual heating began) and at each end point temperature, a sample of the emulsion was removed as described previously. This sample was placed in a preweighed glass-stoppered, 125 ml Erlenmeyer flask under a nitrogen atmosphere and cooled to 25°C in an ice bath. The outside of the cooled flask was dried, the flask and contents were weighed, and the weight of the sample at that temperature was calculated.

After the last sample was removed from the 250 ml round bottom flask, the flask was rinsed with small portions of chloroform and the rinse was added to the beaker for recovery of phospholipids. The chloroform in this beaker was evaporated under a hood and finally in a vacuum desiccator. The weight of phospholipids recovered during these procedures was determined.

Lipids were extracted from each sample of the emulsion by the Ostrander and Dugan (1961) modification of the Bligh and Dyer method. After the lipid extraction was completed, the volumes of the chloroform layer and the methanol-water layer were recorded. The two layers were separated in a 500 ml separatory funnel and both extracts were saved for further analysis. The chloroform extract was used for determination of total lipid and phospholipid classes in the sample, and the methanol-water extract was used for analysis of water-soluble phosphorus.

Total lipid in each sample of the phospholipid emulsion was determined in the following way. Three 30 ml aliquots of the chloroform extracts were pipetted into preweighed dry 50 ml beakers. After evaporation of the chloroform under a hood and finally in a vacuum desiccator, the beakers were weighed and the amount of residue determined.

A blank value was determined as follows. Three samples of 20 g of distilled water were extracted by the Ostrander and Dugan (1961) modification of Bligh and Dyer method. Chloroform, methanol and water used in these extractions were from the same sources as the chloroform,

methanol and water used in the extraction of the samples of the phospholipid emulsion. The volume of the chloroform layer was recorded and the chloroform layer was separated from the methanol-water layer as described previously. Each chloroform extract was sampled as described for determination of total lipid in a sample of the phospholipid emulsion and the weight of the contents in the 50 ml beakers after evaporation of the chloroform was determined. The weight of residue per extraction that was not lipid was determined by:

$$\text{Blank} = \frac{\text{ml } CHCl_3 \text{ extract x g residue in 30 ml aliquot}}{30} .$$

The average weight of residue per extraction for a blank was calculated as 0.0341 g.

Total lipid in the sample of heated phospholipid emulsion was calculated by using the data collected in the extraction of the sample of heated emulsion and the blank in the following equation:

$$\text{Total lipid} = \frac{\text{ml } CHCl_3 \text{ extract x g residue in 30 ml aliquot}}{30} - 0.0341.$$

Percent recovery of phospholipid in each heated phospholipid emulsion was determined as shown in Appendix A.

The remainder of the chloroform layer from the extraction of the sample of heated emulsion was used to determine the phospholipid classes. The phospholipids in this portion were fractionated by thin layer chromatographic procedures described by Turkki and Campbell (1967) and the concentrations of the phospholipid classes were determined by the procedure described by Morrison and Campbell (1971).

The increase in water soluble phosphorus in the samples of heated emulsion at end point temperatures was used as a measurement for the amount of phospholipids decomposed during the heating of the emulsion. This measurement was determined as follows. A measured volume (135-160 ml) of the methanol-water extract from the lipid extraction of each sample was evaporated to dryness in a rotary evaporator. The residue was dissolved in 30 ml of distilled water and saved for phosphorus analysis. Water-soluble phosphorus per ml of this solution was determined by Bartlett's microphosphorus procedure as modified by Marinetti (1962). The water soluble phosphorus (μg P/g emulsion) was determined by the equation:

$$\mu g\ P/g\ \text{emulsion} = \frac{A_{sample}\ (\mu g\ P_{standard})(30)(Z)}{A_{standard}\ (\text{wt sample})(Y)}$$

where A_{sample} is the absorbance of the sample at 830 nm; $A_{standard}$ is the absorbance at 830 nm of a standard containing a known quantity of phosphorus per ml solution ($\mu g\ P_{standard}$); wt sample is the weight in grams of the sample of the phospholipid emulsion; Z is the ml of the methanol-water layer in the extraction and Y is the ml of methanol-water dried in the rotary evaporator.

The increase in water soluble phosphorus (μg P/g emulsion) at any end point temperature was determined by subtracting the water soluble phosphorus found at 25°C from the water soluble phosphorus found at that end point temperature. This increase in water soluble phosphorus was assumed to be from the hydrolysis of phospholipids in

the emulsion.[9] Phospholipids were assumed to contain 4 percent phosphorus; therefore, 25 x the increase in water soluble phosphorus content at any end point temperature would be an estimate of the amount of phospholipids hydrolyzed to water soluble phosphorus compounds during heating from 25°C to that end point temperature.

Preparation, heating and evaluation of protein-phospholipid emulsions. Preparation and heating of the protein-phospholipid emulsions were as described previously (pages 37-38). Evaluations of these emulsions are shown in Figure 6 and Figure 7, pages 43 and 44, respectively. The measurements (total lipid, phospholipid classes, water soluble phosphorus) for I, Figure 6 and for VI, Figure 7, and the same measurements on the protein-phospholipid emulsion before heating were made according to the procedures described for evaluation of phospholipid emulsions (page 94) except that a sample of 15 g instead of 20 g was extracted. The increase in water soluble phosphorus in these emulsions at any end point temperature was measured by subtracting the water soluble phosphorus determined on the methanol-water extract of a sample of the emulsion before heating from that determined on the methanol-water extract of a sample of heated emulsion. The estimate of phospholipids hydrolyzed during heating was determined as previously described (page 96).

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[9]The concentration of phospholipids in any emulsion in this investigation was initially 0.8 g phospholipids/100 g emulsion.

The determination of free and bound lipids in association with dispersed or precipitated protein was carried out as follows. Immediately after centrifugation (Figure 7, page 44) and re-emulsification a sample of 30 g of the heated emulsion which contained the dispersed protein was shell frozen in a 500 ml Virtis freeze drying flask by swirling the flask and contents in a dry ice-acetone bath. The sample was stored under nitrogen at -20°C until it was lyophilized. The precipitate slurry which contained the precipitated protein was kept under nitrogen at 6°C until all analyses were completed (III, Figure 7) and then the remainder (IV, Figure 7) was frozen in its container by swirling the container in a dry ice-acetone bath. The container was placed in a 500 ml Virtis freeze drying flask and its contents lyophilized.

Free lipids and bound lipids were extracted from the freeze dried samples by a modification of the method of Chiu and Pomeranz (1966). The modifications were to extract free lipids with petroleum ether for 6 hours instead of 24 hours[10] and to use the Ostrander and Dugan (1961) modification of the Bligh and Dyer method to extract bound lipids.

Free and bound lipid contents of the freeze dried samples were determined gravimetrically. Phospholipid classes of the free lipid and the bound lipid were determined from the petroleum ether extract and the chloroform extract, respectively.

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[10] A preliminary investigation showed that extraction periods longer than 6 hours did not increase the quantity of free lipids extracted.

The free lipid content was obtained by a difference in weight of the freeze dried sample and the extraction thimble before and after extraction with petroleum ether and was expressed as g lipid/g protein in the sample. The extraction thimble was kept under vacuum in a desiccator until the freeze dried sample was placed in it and the combined weight determined. After extraction, the thimble and the freeze dried sample were dried to a constant weight under a vacuum in a desiccator. All phospholipid containing samples were kept under nitrogen at all times and were stored whenever possible at -20°C.

The bound lipids from the freeze dried samples were extracted by cutting the extraction thimble containing the sample into small pieces and dropping the pieces into the methanol prior to the start of the extraction procedure as modified by Ostrander and Dugan (1961). After the volume of the chloroform had been recorded and the chloroform separated from the methanol-water layer, two 50 ml aliquots of the chloroform extract were pipetted into preweighed 50 ml beakers. The chloroform was evaporated under the hood and finally in a vacuum desiccator, and the average weight of total lipid per 50 ml aliquot was determined. Total bound lipid in the samples was calculated by the equation:

$$\text{g bound lipid in sample} = \frac{\text{ml } CHCl_3 \text{ extract (wt lipid in 50 ml)}}{50} - \text{blank.}$$

The bound lipid was expressed as g lipid/g protein by dividing g bound lipid in sample by g protein in the sample.

The blank was determined by extracting an empty extraction thimble by the Ostrander and Dugan (1961) modification of Bligh and Dyer method, determining the volume of the chloroform extract and the weight of residue in 50 ml aliquots as described for the bound lipid determination and making the same calculation as for the sample extracts.

The petroleum ether extract (free lipid) and the chloroform extract (bound lipid) remaining after lipid quantitation were concentrated by rotary evaporation. The phospholipids of the concentrated extracts were fractionated by thin-layer chromatographic procedures described by Turkki and Campbell (1967) and the concentration of the phospholipid classes (with one exception) was determined by the procedure described by Morrison and Campbell (1971). This exception was the determination of phospholipid classes in lipid bound to dispersed protein in heated sarcoplasmic protein-phospholipid emulsions. A densitogram of the thin layer chromatogram of these phospholipid classes (lysolecithin, sphingomyelin, lecithin, cephalin and two ninhydrin-positive products) is shown in Figure 15. The percent of each class (peaks 1, 2, 4 or 5) and of ninhydrin-positive products (peaks 3 and 6 combined) was determined by dividing the number of integrator counts under the respective peak(s) by the total number of integrator counts under all peaks in the densitogram (Figure 15).

Recovery of phospholipid in heated protein-lipid emulsions. Phospholipids were recovered for each heated protein-lipid emulsion in the following way. Each time the micro jar of the Waring blendor was

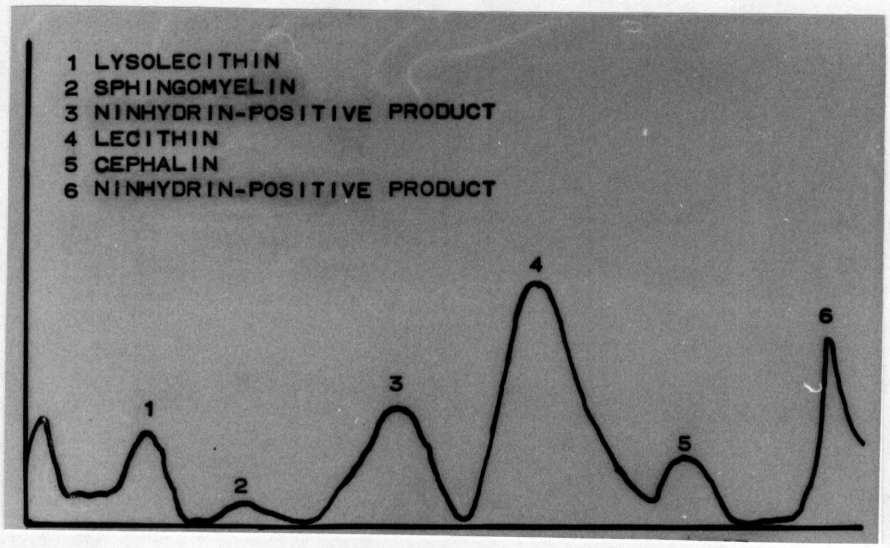


Figure 15. Densitogram of a thin layer chromatogram of phospholipids bound to dispersed protein in a protein-phospholipid emulsion heated to 65°C.

used (preparation and re-emulsification, Figure 7, page 44) it was washed free of lipid in the following manner. Sixty-five ml of methanol were added to the jar and the blendor was operated at a setting of 90 on a Powerstat variable transformer. Thirty-two ml of chloroform were added to the methanol and the solution was blended for one minute. The wash solution was decanted into a stoppered, 500 ml Erlenmeyer flask and was stored under nitrogen at 0°C. This wash solution was used to rinse the following equipment after use to contain or sample the heated emulsion: 250 ml round bottom reaction flask, centrifuge tubes, mortar and pestle, 30 ml Erlenmeyer flask (after removal of freeze-dried precipitate slurry to extraction thimble), pipettes which were used to sample protein slurry for measurements at III, Figure 7, and the pipettes which were used to divide II into V and VI, Figure 7.

Lipids were extracted from the wash solution by a variation of the Ostrander and Dugan (1961) modification of Bligh and Dyer method. The variation was as follows. After the last piece of equipment had been rinsed, the wash solution was blended for 5 minutes in a Waring blendor, setting 40 on a Powerstat variable transformer. Sixty-five ml of a freshly prepared zinc acetate solution containing 1.5 g zinc acetate were added to the blendor and the mixture was blended for 30 more seconds. The extraction procedure followed Ostrander and Dugan's (1961) modification from this point. Total recovered lipid was determined in the following way. After the volume of the chloroform layer had been recorded and the chloroform layer separated from the methanol-

water layer, two 50 ml aliquots of the chloroform extract were placed in preweighed 50 ml beakers. The chloroform was evaporated in a hood and finally in a vacuum desiccator; the beaker and contents were reweighed and the total residue per 50 ml chloroform extract was determined. Total lipid recovered was calculated by the following formula:

$$\frac{\text{ml chloroform extract (weight residue in 50 ml)}}{50} - \text{blank}$$

where the blank was the amount of residue per extraction determined by repeating the recovery operations for a protein-lipid emulsion in which neither the microjar nor the glassware came into contact with any lipid.

Percent recovery of phospholipids was calculated for all heated protein-lipid emulsions and a sample of calculation is shown in Appendix B.

Mathematical and statistical procedure. The quantity of each phospholipid class (bound or free) in association with protein (dispersed or precipitated) was calculated by multiplying the quantity of lipid (bound or free) in association with protein (dispersed or precipitated) by the percent of the phospholipid class in the lipid.

Bound and free lipid and the quantity of each phospholipid class in association with total protein in the heated protein-lipid emulsions were calculated the same way as the measurements for total protein (page 45). The measurements for quantity of lipid and each phospholipid class were expressed as g lipid/g protein.

A two-way analysis of variance with replication for a Model-I Anova was used for analysis of all data. The Student-Newman-Keuls

test was used to identify significant differences between means (Sokal and Rohlf, 1969).

Results and Discussion

Percent recoveries of phospholipids and percent phospholipid classes in heated phospholipid and protein-phospholipid emulsions. Tables for the recovery of phospholipids in the heated phospholipid emulsions and in the heated protein-phospholipid emulsions are shown in Table 31, Appendix A, and Table 32, Appendix C, respectively. The percent phospholipid classes of heated phospholipid emulsions are shown in Table 33, Appendix D. The percent phospholipid classes of free and bound lipid in association with dispersed and precipitated protein in heated sarcoplasmic protein-phospholipid emulsions and in heated myoglobin-phospholipid emulsions are shown in Tables 34 and 35, Appendix D, respectively.

Decomposition of phospholipids by heating. Shown in Table 17 are the amounts of phospholipids decomposed during the heating of phospholipid emulsions and sarcoplasmic protein-phospholipid emulsions. Significant differences were not observed for quantity of phospholipid decomposed with increasing temperature because of wide differences in the values of the duplicates in the heated sarcoplasmic protein-phospholipid emulsions. A significant difference was found at 77°C between the phospholipid emulsion and the sarcoplasmic protein-phospholipid emulsion. A significantly larger amount of phospholipid was destroyed by

TABLE 17

QUANTITY OF PHOSPHOLIPIDS DECOMPOSED TO WATER SOLUBLE PHOSPHORUS COMPOUNDS BY HEATING PHOSPHOLIPID EMULSIONS AND SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

Emulsion	Temperatures to Which Emulsions Were Heated				
	25°C	56°C	60°C	65°C	77°C
	----μg phospholipids decomposed/g emulsion----				
Phospholipid	0.00[a]	27.8[a]	34.0[a]	46.4[a]	90.8[a]
Sarcoplasmic protein-phospholipid	0.00[a,b]	8.0[a,b]	36.0[a,b]	12.8[a,b]	-15.2[b]

[a,b]Values in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

heating in the phospholipid emulsion than in the sarcoplasmic protein-phospholipid emulsion.

Table 18 shows the amounts of phospholipid decomposed by heating all three emulsions: phospholipid, sarcoplasmic protein-phospholipid, and myoglobin-phospholipid. Significant amounts of phospholipids were decomposed to water soluble phosphorus compounds by heating the phospholipid emulsion to 77°C and by heating the myoglobin-phospholipid emulsion to 65 or 77°C. Heating the sarcoplasmic protein-phospholipid emulsion caused no decomposition of the phospholipids to water soluble phosphorus compounds. Decomposition of phospholipids caused by heating will be discussed later in this chapter. The negative value for the heated sarcoplasmic-phospholipid emulsions at 77°C (Table 18) indicates that more water soluble phosphorus was found in the sarcoplasmic protein-phospholipid emulsion before heating than after heating.

Quantity of phospholipid in association with protein in heated protein-lipid emulsions. The quantity of phospholipids (bound and free) in association with total protein in heated sarcoplasmic protein-phospholipid emulsions is given in Table 19. No significant differences were observed with increasing temperature or between bound and free lipid.

The quantity of bound and free phospholipids in association with dispersed and precipitated protein in heated sarcoplasmic protein-phospholipid emulsions is reported in Table 20. Increasing the temperature from 25 to 56°C significantly decreased the amount of free lipid

TABLE 18

QUANTITY OF PHOSPHOLIPIDS DECOMPOSED TO WATER SOLUBLE PHOSPHORUS COMPOUNDS IN THE FOLLOWING HEATED EMULSIONS: PHOSPHOLIPID, SARCOPLASMIC PROTEIN-PHOSPHOLIPID AND MYOGLOBIN-PHOSPHOLIPID

Emulsion	Temperatures to Which Emulsions Were Heated		
	25°C	65°C	77°C
	--µg phospholipids decomposed/g emulsion---		
Phospholipid	0.00[a]	46.4[a,b]	90.8[b]
Sarcoplasmic protein-phospholipid	0.00[a]	12.8[a,b]	-15.2[a]
Myoglobin-phospholipid	0.00[a]	79.5[b]	82.5[b]

[a,b]Values in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

TABLE 19

QUANTITY OF PHOSPHOLIPIDS (BOUND AND FREE) IN ASSOCIATION WITH TOTAL PROTEIN IN HEATED SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

	Temperatures[a] to Which Emulsions Were Heated				
	25°C	56°C	60°C	65°C	77°C
	g lipid/g protein				
Bound lipid[a]	0.2691	0.4136	0.3163	0.2856	0.3465
Free lipid[a]	0.3102	0.2611	0.2312	0.2384	0.2370

[a]Significant differences were not found between bound and free lipid or among temperatures at the $P < 0.05$ level.

TABLE 20

QUANTITY OF PHOSPHOLIPIDS (BOUND AND FREE) IN ASSOCIATION WITH DISPERSED AND PRECIPITATED PROTEIN IN HEATED SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

State of Protein	Temperatures to Which Emulsions Were Heated				
	25°C	56°C	60°C	65°C	77°C
	g lipid/g protein				
Dispersed protein					
Bound lipid	0.2691[a,b]	0.4019[a]	0.2204[a,c]	0.1908[a,c]	0.2633[a]
Free lipid	0.3102[b]	0.1467[c]	0.0931[c]	0.0752[c]	0.0879[c]
Precipitated protein					
Bound lipid	0.0000[c]	0.4356[a,d]	0.4858[d]	0.4488[d]	0.4736[d]
Free lipid	0.0000[c]	0.4504[a,d]	0.4694[d]	0.5261[d]	0.4638[d]

[a,b,c,d]Values in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

associated with dispersed protein in the emulsion. The significant difference between 25 and 56°C for bound and free lipid in association with precipitated protein shows only that protein with free and bound lipid did precipitate as the emulsion was heated. At 60, 65 and 77°C there is significantly more lipid, bound or free, in association with precipitated protein than with dispersed protein. At 56 and 77°C there is significantly more bound than free lipid in the dispersed protein.

The quantity of phospholipid, bound and free, in association with total protein in heated myoglobin-phospholipid emulsions is shown in Table 21. Significant differences were not observed with increasing temperature or between bound and free lipid.

The quantity of phospholipids in association with dispersed and precipitated protein in heated myoglobin-phospholipid emulsions is reported in Table 22. At 77°C the quantity of phospholipids bound to the precipitated protein was significantly larger than the quantity of free phospholipids in association with the precipitated protein and the quantities of lipids (bound or free) in association with dispersed protein. The significant temperature-lipid association interaction in Table 22 reflects the tendency for lipid bound to precipitated protein to increase with increasing temperature whereas free lipid did not increase after 65°C. During heating either the protein that is associated with larger quantities of phospholipids (bound or free) is precipitated or the protein that is precipitated by heating becomes associated with larger quantities of phospholipids (bound or free).

TABLE 21

QUANTITY OF PHOSPHOLIPIDS (BOUND AND FREE) IN ASSOCIATION WITH TOTAL PROTEIN IN HEATED MYOGLOBIN-PHOSPHOLIPID EMULSIONS

Lipid Association	Temperatures[a] to Which Emulsions Were Heated		
	25°C	65°C	77°C
	g lipid/g protein		
Bound lipid[a]	1.0060	1.7966	1.7282
Free lipid[a]	0.7224	1.4124	1.0323

[a]Significant differences between values were not found at the $P < 0.05$ level.

TABLE 22

QUANTITY OF PHOSPHOLIPIDS (BOUND AND FREE) IN ASSOCIATION WITH DISPERSED AND PRECIPITATED PROTEIN IN HEATED MYOGLOBIN-PHOSPHOLIPID EMULSIONS

State of Protein Lipid Association[d]	Temperatures[d] to Which Emulsions Were Heated		
	25°C	65°C	77°C
	g lipid/g protein		
Dispersed Protein			
Bound lipid	1.0060[a]	1.5145[a,b]	0.8965[a]
Free lipid	0.7224[a]	1.0090[a,b]	0.6794[a]
Precipitated Protein			
Bound lipid	0.0000[a]	2.7430[b,c]	4.7015[c]
Free lipid	0.0000[a]	3.1396[b]	2.0448[a,b]

[a,b,c]Values in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

[d]The temperature-lipid association interaction is significant at the $P < 0.05$ level.

The quantities of phospholipid classes in lipid, bound and free, that are associated with total protein in heated sarcoplasmic protein-phospholipid emulsions are reported in Table 23. There was only one significant difference in all classes with increasing temperature. The quantity of lecithin in the bound lipid at 56°C was significantly larger than the quantity of lecithin in bound lipid at any other temperature. At all temperatures significantly larger quantities of lecithin than of any other phospholipid class were found in the bound lipid. This observation may be made also for the phospholipid classes of the free lipid except that at 25, 60, 65 and 77°C the quantities of lecithin in the free lipid were not significantly different from the quantities of cephalin. The only significant difference observed in quantities of a single phospholipid class between bound and free lipid was for lysolecithin. At all temperatures significantly larger quantities of lysolecithin were found in the bound lipid than were found in the free lipid.

Quantities of phospholipid classes in association with protein in heated protein-lipid emulsions. Quantities of phospholipid classes in bound and free lipid associated with dispersed protein in heated sarcoplasmic protein-phospholipid emulsions are reported in Table 24. The significant differences observed with increasing temperature were: (1) a decrease for lecithin in the bound lipid between the quantities at 25 and 56°C and the quantities at 60, 65 and 77°C; (2) an increase in the ninhydrin positive products in the bound lipid from the quantity

TABLE 23

QUANTITIES OF PHOSPHOLIPID CLASSES IN BOUND AND FREE LIPID ASSOCIATED WITH TOTAL PROTEIN IN HEATED SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

Lipid Association Phospholipid Class	Temperatures to Which Emulsions Were Heated				
	25°C	56°C	60°C	65°C	77°C
	g lipid/g protein				
Bound Lipid					
Lysolecithin[d]	0.0198[a]	0.0345[a]	0.0329[a]	0.0218[a]	0.0463[a]
Sphingomyelin[e]	0.0194[a]	0.0228[a]	0.0221[a]	0.0184[a]	0.0240[a]
Lecithin[e]	0.1776[b]	0.2373[c]	0.1803[b]	0.1655[b]	0.1613[b]
Cephalin[e]	0.0506[a]	0.0672[a]	0.0470[a]	0.0488[a]	0.0559[a]
Ninhydrin-positive products	0.0018[a]	0.0519[a]	0.0341[a]	0.0311[a]	0.0590[a]
Free Lipid					
Lysolecithin[d]	0.0095[a]	0.0060[a]	0.0085[a]	0.0047[a]	0.0106[a]
Sphingomyelin[e]	0.0372[a]	0.0254[a]	0.0246[a]	0.0272[a]	0.0294[a]
Lecithin[e]	0.1698[b]	0.1691[b]	0.1373[b]	0.1384[b]	0.1321[b]
Cephalin[e]	0.0937[a,b]	0.0606[a]	0.0606[a,b]	0.0685[a,b]	0.0648[a,b]

[a,b,c]For any one lipid association quantities in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

[d]Quantities of lysolecithin in bound and unbound lipid are significantly different at the $P < 0.05$ level at all temperatures.

[e]Quantities in bound and unbound lipid are not significantly different at the $P < 0.05$ level for any temperature.

TABLE 24

QUANTITIES OF PHOSPHOLIPID CLASSES IN BOUND AND FREE LIPID ASSOCIATED WITH DISPERSED PROTEIN IN HEATED SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

Lipid Association Phospholipid Class[d]	Temperatures[d] to Which Emulsions Were Heated				
	25°C	56°C	60°C	65°C	77°C
	g lipid/g protein				
Bound Lipid					
Lysolecithin	0.0198[a]	0.0368[a,b]	0.0303[a]	0.0188[a]	0.0440[a,b]
Sphingomyelin	0.0194[a]	0.0164[a,b]	0.0120[a]	0.0085[a]	0.0117[a]
Lecithin	0.1776[c]	0.1967[c]	0.1055[b]	0.0966[b]	0.0748[a,b]
Cephalin	0.0506[a]	0.0681[a,b]	0.0190[a]	0.0179[a]	0.0349[a,b]
Ninhydrin-positive products	0.0018[a]	0.0840[b]	0.0537[a,b]	0.0490[a,b]	0.0979[b]
Free Lipid					
Lysolecithin	0.0095[a]	0.0000[a]	0.0048[a]	0.0014[a]	0.0041[a]
Sphingomyelin	0.0372[a]	0.0000[a]	0.0059[a]	0.0052[a]	0.0000[a]
Lecithin	0.1698[c]	0.1243[b,c]	0.0734[a,b]	0.0572[a,b]	0.0505[a,b]
Cephalin	0.0937[a]	0.0224[a]	0.0090[a]	0.0118[a]	0.0334[a]

[a,b,c]Quantities for each lipid association in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

[d]The temperature-phospholipid class interaction for bound lipid is significant at the $P < 0.01$ level.

at 25°C to the quantities at 56 and 77°C; and (3) a decrease in lecithin in the free lipid between the quantity at 25°C and the quantities at 60, 65 and 77°C. Significantly larger quantities of lecithin were found in bound or free lipid at 25 and 56°C than of any other phospholipid class in bound or free lipid at the same temperature.

The ninhydrin-positive products were determined from the combined areas of peaks 3 and 6, Figure 15, page 101. These products were not originally present in the phospholipids used to prepare the emulsion and therefore are probably decomposition products of the phospholipids caused by heating of the emulsion. The spots of the chromatogram represented by peaks 3 and 6 gave a positive ninhydrin and negative Dragendorf test. Turkki (1965) reported that a synthetic dipalmitoyl phosphatidyl ethanolamine (a cephalin) when separated by thin layer chromatographic procedure of Campbell and Turkki (1967) gave three ninhydrin-positive spots,[11] two of which probably represented different geometric isomers and one, a lysocephalin. In the present investigation peak 3 probably represents the lysocompound and peak 6 probably represents a geometric isomer of cephalin. These products must be considered to be decomposition products of cephalin caused by heating the emulsion.

The binding of decomposition products of phospholipid to the dispersed protein of heated sarcoplasmic protein-phospholipid emulsions must be at least partly responsible for the fact that no phospholipids

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[11]These spots also gave a negative Dragendorf test.

were found to be decomposed to water soluble phosphorus compounds in heated sarcoplasmic protein-phospholipid emulsions (Table 18, page 107). The emulsion before heating contained 14 mg protein/g emulsion (page 39) and after heating 60.4 percent of the protein remained dispersed (Table 5, page 47). If the following equation is solved,

$$\mu g \text{ decomposition products/g emulsion} = \left(\frac{97.9\ \mu g \text{ products}}{\text{mg dispersed protein}}\right)\left(\frac{14 \text{ mg protein}}{\text{g emulsion}}\right)\left(\frac{60.4 \text{ mg dispersed protein}}{100 \text{ mg protein}}\right)$$

it can be seen that heating the sarcoplasmic protein-phospholipid emulsion from 25 to 77°C caused 827 μg phospholipid/g emulsion to be altered to decomposition products. This amount is considerably more than the 90 μg phospholipids/g emulsion decomposed to water soluble phosphorus compounds in phospholipid emulsions heated from 25 to 77°C (Table 18). Ninhydrin-positive products were not found in the heated phospholipid emulsion (Appendix D). These results seem to agree with the results reported by Nakanishi and Kaya (1970b) who found that there was a greater rate of decomposition of phospholipids heated in milk (a dispersion containing proteins) than of phospholipids heated in water.

Quantities of phospholipid classes in the bound and free lipid associated with precipitated protein of heated sarcoplasmic protein-phospholipid emulsions are reported in Table 25. Significant differences were not observed among temperatures for any of the phospholipid classes in the bound or free lipid. At all temperatures in bound and free lipid there were significantly larger quantities of lecithin than of any other phospholipid classes. At 60 and 65°C in bound and free

TABLE 25

QUANTITIES OF PHOSPHOLIPID CLASSES IN BOUND AND FREE LIPID ASSOCIATED WITH PRECIPITATED PROTEIN IN HEATED SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

Lipid Association Phospholipid Class	Temperatures to Which Emulsions Were Heated 56°C	60°C	65°C	77°C
	g lipid/g protein			
Bound Lipid				
Lysolecithin	0.0317[a]	0.0376[a]	0.0271[a]	0.0499[a]
Sphingomyelin	0.0330[a]	0.0409[a]	0.0361[a]	0.0426[a]
Lecithin	0.3052[b]	0.3107[b]	0.2929[b]	0.2933[b]
Cephalin	0.0657[a,c]	0.0967[c]	0.0926[c]	0.0879[a,c]
Free Lipid				
Lysolecithin	0.0158[a]	0.0148[a]	0.0104[a]	0.0206[a]
Sphingomyelin	0.0676[a,b]	0.0559[a]	0.0658[a]	0.0741[a,b]
Lecithin	0.2433[c]	0.2491[c]	0.2820[c]	0.2563[c]
Cephalin	0.1237[b]	0.1498[b]	0.1680[b]	0.1128[b]

[a,b,c]Quantities for one lipid association in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

lipid there were significantly larger quantities of cephalin than of lysolecithin or sphingomyelin.

The quantity of each phospholipid class in bound and free lipid that is in association with dispersed and precipitated protein in heated sarcoplasmic protein-phospholipid emulsions is reported in Table 26. Analysis of variance of the rearranged data, previously reported in Table 24, page 115, and Table 25, show whether or not a phospholipid class was found in bound or free lipid that was in association with a particular state of protein (dispersed or precipitated) in the emulsions.

For lysolecithin in Table 26 the following significant differences were observed. At 56 and 77°C significantly larger quantities of lysolecithin were in the lipid bound to dispersed protein than in the free lipid associated with dispersed or precipitated protein. At 60, 65 and 77°C significantly larger quantities of lysolecithin were in the lipid bound to the precipitated protein than in the free lipid of dispersed or precipitated protein. Significant increases in lysolecithin bound to dispersed and precipitated protein were observed between 25°C and all other temperatures with one exception: lysolecithin bound to dispersed protein at 65°C. These observations indicate that in heated emulsions larger quantities of lysolecithin became associated with protein by polar interactions (Fullington, 1969).

Significant differences also were observed for quantities of sphingomyelin (Table 26). In emulsions heated to 60, 65 and 77°C

TABLE 26

QUANTITIES OF EACH PHOSPHOLIPID CLASS IN BOUND AND FREE LIPID IN DISPERSED AND PRECIPITATED PROTEIN IN HEATED SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

Phospholipid Class State of Protein Lipid Association[f]	Temperatures[f] to Which Emulsions Were Heated				
	25°C	56°C	60°C	65°C	77°C
	g lipid/g protein				
Lysolecithin					
Dispersed protein					
Bound lipid	0.0198[a]	0.0368[b,c]	0.0303[b,c]	0.0188[a,b]	0.0440[b,c]
Free lipid	0.0095[a,b]	0.0000[a]	0.0048[a]	0.0014[a]	0.0041[a]
Precipitated protein					
Bound lipid	0.0000[a]	0.0317[d,c]	0.0376[c]	0.0271[c]	0.0499[c]
Free lipid	0.0000[a]	0.0158[a,d]	0.0148[a,b]	0.0104[a]	0.0206[a]
Sphingomyelin					
Dispersed protein					
Bound lipid	0.0194[a,b]	0.0164[a,c]	0.0120[a]	0.0085[a]	0.0117[a]
Free lipid	0.0372[a]	0.0000[a]	0.0059[a]	0.0052[a]	0.0000[a]
Precipitated protein					
Bound lipid	0.0000[b]	0.0330[c]	0.0409[c]	0.0361[c]	0.0426[c]
Free lipid	0.0000[b]	0.0676[d]	0.0559[c,d]	0.0658[d]	0.0741[d]
Lecithin					
Dispersed protein					
Bound lipid	0.1776[a]	0.1967[a,c]	0.1055[a]	0.0966[a]	0.0748[a]
Free lipid	0.1698[a]	0.1243[a]	0.0734[a]	0.0572[a]	0.0505[a]
Precipitated protein					
Bound lipid	0.0000[b]	0.3052[d]	0.3107[d]	0.2929[d]	0.2933[d]
Free lipid	0.0000[b]	0.2433[c,d]	0.2491[d]	0.2820[d]	0.2563[d]
Cephalin					
Dispersed protein					
Bound lipid	0.0506[a,b]	0.0681[a]	0.0190[a]	0.0179[a]	0.0349[a,c]
Free lipid	0.0937[b]	0.0224[a]	0.0090[a]	0.0118[a]	0.0334[a]
Precipitated protein					
Bound lipid	0.0000[c]	0.0657[a,e]	0.0967[e]	0.0926[e]	0.0879[d,e]
Free lipid	0.0000[c]	0.1237[d]	0.1498[d]	0.1680[d]	0.1128[d]
Ninhydrin-positive Products					
Dispersed protein					
Bound lipid	0.0018[a]	0.0840[a]	0.0537[a]	0.0490[a]	0.0979[a]

[a,b,c,d,e]Values in the same row or column for one phospholipid class bearing like superscript letters are not significantly different ($P < 0.05$).

[f]The temperature-lipid association interaction for any one phospholipid class (lysolecithin, sphingomyelin, lecithin, cephalin) was significant at the $P < 0.05$ level.

there were significantly larger quantities of sphingomyelin in the bound and free lipid associated with precipitated protein than in bound or free lipid associated with dispersed protein. At 65 and 77°C significantly larger quantities of sphingomyelin were found in the free lipid associated with the precipitated protein than in the lipid bound to the precipitated protein. The only significant difference noted among temperatures was the increase in the quantity of sphingomyelin associated with precipitated protein between 25°C and all other temperatures which indicates that a precipitate containing protein in association with bound and free sphingomyelin was formed as the emulsions were heated.

The following significant differences for quantities of lecithin in the bound and free lipid in association with dispersed and precipitated protein were observed (Table 26). At 60, 65 and 77°C significantly larger quantities of lecithin were found in lipid (bound and free) associated with the precipitated protein than in lipid (bound and free) associated with dispersed protein. At 56°C a significantly larger quantity of lecithin was found bound to precipitated protein than was found either bound or free in association with the dispersed protein. The significant increase in the quantities of lecithin associated with precipitated protein between 25°C and all other temperatures indicates that a precipitate containing protein in association with lecithin was formed when the emulsion was heated.

At 56, 60 and 65°C significantly larger quantities of cephalin (Table 26) were in the free lipid associated with precipitated protein

than were in the lipid bound to the precipitated protein or in the lipid (bound or free) associated with dispersed protein. At 60, 65 and 77°C significantly larger quantities of cephalin were found in lipid (bound or free) associated with precipitated protein than in lipid (bound or free) associated with dispersed protein. A significant decrease for cephalin in free lipid associated with the dispersed protein was observed between the quantity at 25°C and the quantities at all temperatures. The significant differences between 25°C and all temperatures for cephalin in bound or free lipid associated with precipitated protein indicates that a precipitate containing protein associated with cephalin was formed as the emulsions were heated.

In heated emulsions there was a partitioning of sphingomyelin, lecithin and cephalin in which a larger part of each phospholipid was associated with precipitated protein than with dispersed protein (Table 26, page 120). There also were differences in the quantities of two of these phospholipids between bound and free lipid associated with precipitated protein. Significantly larger quantities of sphingomyelin and cephalin were found in the free lipid than in the bound lipid, but lecithin was distributed fairly evenly between bound and free lipid. Free lipid is associated with protein either by occlusion (remaining unbound) or by hydrophobic bonds (Hoseney et al., 1970).

The significant temperature-lipid association interaction for any one phospholipid class (Table 26) is attributable to the variations among the quantities of free and bound lipid in association with protein with increasing temperature.

Quantities of phospholipid classes of bound and free lipid in association with total protein of heated myoglobin-phospholipid emulsions are shown in Table 27. There were significantly larger quantities of lecithin than of any other phospholipid class in bound and free lipid at each temperature. A significant increase for lecithin was observed: (1) in bound lipid between 25°C and each of the other temperatures and (2) in free lipid between 25 and 65°C.

Quantities of phospholipid classes in bound and free lipid associated with dispersed protein are shown in Table 28. Only lecithin of bound lipid differed significantly in extent of association at different temperatures.

Quantities of phospholipid classes in bound and free lipid associated with precipitated protein in heated myoglobin-phospholipid emulsions are shown in Table 29. There were significantly larger quantities of lecithin than of any other phospholipid class in the bound and free lipid associated with precipitated protein. Significant differences were not observed with increasing temperature.

Quantities of each phospholipid class in bound and free lipid associated with dispersed and precipitated protein in heated myoglobin-phospholipid emulsions are reported in Table 30. These data are rearranged data previously reported in Tables 28 and 29 (see explanation for Table 26, page 119). The significant increases in quantities of lysolecithin, sphingomyelin, lecithin and cephalin in bound or free lipid associated with precipitated protein with heating from 25°C to the higher temperatures indicate that protein associated with these

TABLE 27

QUANTITIES OF PHOSPHOLIPID CLASSES IN BOUND AND FREE LIPID ASSOCIATED WITH TOTAL PROTEIN IN HEATED MYOGLOBIN-PHOSPHOLIPID EMULSIONS

Lipid Association Phospholipid Class	Temperatures to Which Emulsions Were Heated		
	25°C	65°C	77°C
	g lipid/g protein		
Bound Lipid			
Lysolecithin	0.0999[a]	0.1443[a]	0.1939[a]
Sphingomyelin	0.0544[a]	0.1915[a]	0.1735[a]
Lecithin	0.6738[b]	1.1269[c]	1.0803[c]
Cephalin	0.1428[a]	0.2719[a]	0.2805[a]
Ninhydrin-positive products	0.0351[a]	0.0621[a]	0.0000[a]
Free Lipid			
Lysolecithin	0.0345[a]	0.0553[a]	0.0552[a]
Sphingomyelin	0.0992[a]	0.1740[a]	0.1650[a]
Lecithin	0.4817[b,d]	0.9958[c]	0.6824[d]
Cephalin	0.1070[a]	0.1874[a]	0.1297[a]

[a,b,c,d]Quantities in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

TABLE 28

QUANTITIES OF PHOSPHOLIPID CLASSES IN BOUND AND FREE LIPID ASSOCIATED WITH DISPERSED PROTEIN IN HEATED MYOGLOBIN-PHOSPHOLIPID EMULSIONS

Lipid Association Phospholipid Class[d]	Temperatures[d] to Which Emulsions Were Heated		
	25°C	65°C	77°C
	g lipid/g protein		
Bound Lipid			
Lysolecithin	0.0999[a]	0.1276[a]	0.1775[a]
Sphingomyelin	0.0544[a]	0.1491[a]	0.1135[a]
Lecithin	0.6736[b]	0.9533[c]	0.4421[d]
Cephalin	0.1428[a]	0.1892[a]	0.1634[a]
Ninhydrin-positive products	0.0351[a]	0.0953[a]	0.0000[a]
Free Lipid			
Lysolecithin	0.0345[a]	0.0290[a]	0.0321[a,b]
Sphingomyelin	0.0992[a]	0.1050[a]	0.0938[a]
Lecithin	0.4817[a,b]	0.7965[b]	0.4789[b]
Cephalin	0.1070[a]	0.0787[a]	0.0749[a,b]

[a,b,c]Quantities for one lipid association in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

[d]The temperature-phospholipid class interaction for bound lipid is significant at the $P < 0.01$ level.

TABLE 29

QUANTITIES OF PHOSPHOLIPID CLASSES IN BOUND AND FREE LIPID ASSOCIATED WITH PRECIPITATED PROTEIN IN HEATED MYOGLOBIN-PHOSPHOLIPID EMULSIONS

Lipid Association Phospholipid Class	Temperatures to Which Emulsions Were Heated 65°C	 77°C
	g lipid/g protein	
Bound Lipid		
Lysolecithin	0.2462[a]	0.1227[a]
Sphingomyelin	0.5338[a]	0.3720[a]
Lecithin	1.8477[b]	1.2651[a,b]
Cephalin	0.5119[a]	0.2848[a]
Free Lipid		
Lysolecithin	0.2160[a]	0.2527[a]
Sphingomyelin	0.3239[a]	0.3974[a]
Lecithin	1.7655[b]	3.3653[b]
Cephalin	0.4377[a]	0.6865[a]

[a,b]Quantities for one lipid association in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

TABLE 30

QUANTITIES OF EACH PHOSPHOLIPID CLASS IN BOUND AND FREE LIPID ASSOCIATED WITH DISPERSED AND PRECIPITATED PROTEIN IN HEATED MYOGLOBIN-PHOSPHOLIPID EMULSIONS

Phospholipid Class State of Protein Lipid Association[d]	Temperatures[d] to Which Emulsions Were Heated 25°C	65°C	77°C
	g lipid/g protein		
Lysolecithin			
Dispersed protein			
Bound lipid	0.0999[a]	0.1276[a,b]	0.1775[a,b]
Free lipid	0.0345[a]	0.0290[a,b]	0.0321[a,b]
Precipitated protein			
Bound lipid	0.0000[a]	0.2160[b]	0.2527[b]
Free lipid	0.0000[a]	0.2462[b]	0.1227[a,b]
Sphingomyelin			
Dispersed protein			
Bound lipid	0.0544[a]	0.1491[a,c]	0.1135[a,c]
Free lipid	0.0992[a]	0.1050[a,c]	0.0938[a,c]
Precipitated protein			
Bound lipid	0.0000[a]	0.3239[a,c]	0.3974[a,c]
Free lipid	0.0000[a]	0.5338[c]	0.3720[c]
Lecithin			
Dispersed protein			
Bound lipid	0.6736[a]	0.9533[a,b]	0.4221[a,c]
Free lipid	0.4817[a]	0.7965[a,b]	0.4789[a,c]
Precipitated protein			
Bound lipid	0.0000[a]	1.7655[b]	3.3653[c]
Free lipid	0.0000[a]	1.8477[a,b]	1.26505[a,c]
Cephalin			
Dispersed protein			
Bound lipid	0.1428[a]	0.1892[a,b]	0.1634[a]
Free lipid	0.1070[a]	0.0787[a]	0.0749[a]
Precipitated protein			
Bound lipid	0.0000[a]	0.4377[b,c]	0.6865[b]
Free lipid	0.0000[a]	0.5119[c]	0.2848[a,c]
Ninhydrin-positive Products			
Dispersed protein			
Bound lipid	0.0351[a]	0.0953[a]	0.0000[a]

[a,b,c]Quantities for each phospholipid class in the same row or column bearing like superscript letters are not significantly different at the $P < 0.05$ level.

[d]The temperature-lipid association interaction for lecithin and cephalin were significant at the $P < 0.05$ level.

phospholipid classes precipitated when the emulsions were heated. Quantities of sphingomyelin and lecithin in bound and free lipid associated with precipitated protein were larger than the quantities in bound and free lipid associated with dispersed proteins, but they were not significantly larger. In many instances in the heated myoglobin-phospholipid emulsions there were wide variations between duplicate values for quantities of phospholipid classes in the bound or free lipid associated with a particular state of protein, and this variation is the reason that the large differences between means in Table 30 were not significant. At 65°C there was a significantly larger quantity of cephalin found in free lipid associated with precipitated protein than was found in bound or free lipid associated with dispersed protein. At 77°C a significantly larger quantity of cephalin was found in lipid bound to precipitated protein than quantities found in any other lipid-protein association. These trends and differences where significant tend to support the indications seen in Table 26, page 120, with the exception that in the myoglobin-phospholipid emulsions at 77°C cephalin apparently was associated preferentially with the precipitated protein by polar interaction (bound lipid).

The significant temperature-lipid association interaction for lecithin and cephalin (Table 30) is attributable to the variations among free and bound lipid in association with dispersed or precipitated protein with increasing temperature.

Peak 6[12] (Figure 15, page 101) accounted for the quantities of ninhydrin-positive products that were found in lipid bound to total or dispersed protein reported in Table 27, page 124, Table 28, page 125, and Table 30, page 127. This peak was assumed to be a geometric isomer of cephalin as previously described (page 116). There was no known reason why this compound was found in myoglobin-phospholipid emulsions at 25°C (Tables 27, 28 and 30); it was not present in the phospholipids prior to emulsification. It tended to increase in the myoglobin-phospholipid emulsions heated to 65°C and to disappear in the emulsions heated to 77°C; however, these differences were not significant.

The quantities of bound and free phospholipid associated with total dispersed or precipitated protein in myoglobin-phospholipid emulsions were large compared to the quantities of bound and free lipid associated with the protein in sarcoplasmic protein-phospholipid emulsions. Gulik-Krzywicki et al. (1969) used X-ray diffraction to investigate systems of protein-phospholipid-water and found that the X-ray diffraction diagrams were typical of a lamellar structure of protein-lipid-protein-lipid-protein etc. In systems where lipid was in excess of protein, the thickness of the lipid layer shrunk extensively as compared with the thickness of a lipid layer in a system in which protein was in excess of lipid. Possibly, more lipid would be associated with the protein in an emulsion in which the lipid was in excess of protein than in an emulsion in which the protein was in

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[12]Peak 3 which was also a ninhydrin-positive product was not found in the lipid analyses of myoglobin emulsions.

excess of the lipid. In the phospholipid-myoglobin emulsions in the present investigation, the phospholipids were in excess of myoglobin (0.8 g phospholipids/100 g emulsion compared with 0.2 g myoglobin/100 g emulsion) and the observation reported by Gulik-Krzywicki et al. (1969) was probably the reason for the large quantities of phospholipids associated with the protein.

Summary

Phospholipids and sarcoplasmic proteins were extracted from bovine ground round and myoglobin was prepared from sarcoplasmic protein extracts by ammonium sulfate fractionation. Phospholipid emulsions and sarcoplasmic protein-phospholipid emulsions were heated from 25°C to end point temperatures of 56, 60, 65 and 77°C at a rate of temperature increase of 1°C/min. Myoglobin-phospholipid emulsions were heated from 25 to 65 and 77°C at the same rate. All emulsions initially contained 0.8 g phospholipids/100 g emulsion. The sarcoplasmic protein emulsions initially contained 1.4 g protein/100 g emulsion and the myoglobin emulsions initially contained 0.2 g protein/100 g emulsion. The following measurements were conducted on protein-containing emulsions: (1) quantities of free and bound lipid associated with dispersed, precipitated and total protein in the emulsions and (2) quantities of phospholipid classes (lysolecithin, sphingomyelin, lecithin, cephalin, ninhydrin-positive products) in free and bound lipid associated with each protein described in (1). Increase in water-soluble phosphorus that was used to determine decomposition

of phospholipids to water soluble phosphorus compounds was determined on all emulsions.

Heating the phospholipid or myoglobin-phospholipid emulsion to 77°C caused approximately 1 percent of the total phospholipids to decompose to water soluble phosphorus compounds and this decomposition was significant at the $P < 0.05$ level. Heating the sarcoplasmic protein-phospholipid emulsion did not cause significant increases in water-soluble phosphorus compounds; however, ninhydrin-positive products which were tentatively identified as heat decomposition products of cephalin were found bound to the dispersed protein in heated sarcoplasmic protein emulsions. These compounds represented approximately a 10 percent decomposition of the total phospholipid in the emulsion, and the quantity of these compounds found in emulsions heated to 77°C was significantly larger than the quantity found in emulsions at 25°C ($P < 0.05$).

Heating the sarcoplasmic protein-phospholipid emulsions did not affect the amount of free or bound lipid associated with total protein. However, when the total protein was separated into dispersed and precipitated protein in heated sarcoplasmic protein emulsions, heating to temperatures above 56°C caused free lipid (unbound and hydrophobically-bound) associated with dispersed protein to decrease significantly ($P < 0.05$). When the free and bound lipid associated with dispersed protein in sarcoplasmic protein emulsions was separated into phospholipid classes, heating generally (1) decreased significantly ($P < 0.05$) the quantity of lecithin in free or bound lipid; (2) decreased

significantly ($P < 0.05$) the quantity of cephalin in free lipid and (3) increased significantly ($P < 0.05$) the quantities of lysolecithin and ninhydrin-positive products in the bound lipid. Heating the sarcoplasmic protein emulsions from 56 to 77°C did not affect significantly either the quantity of bound and free lipid associated with the precipitated protein or the quantity of the phospholipid classes in the bound or free lipid. However, heating the emulsion from 25 to 56°C caused formation of a precipitate containing protein associated with both types lipid in which all phospholipid classes were represented.

Heating the myoglobin-phospholipid emulsions did not affect significantly the quantity of free or bound lipid associated with total or dispersed protein. When the free and bound lipids were separated into phospholipid classes, heating from 25 to 65°C caused a significant increase ($P < 0.05$) in the amount of lecithin in free and bound lipid associated with total or dispersed protein. When the temperature was increased from 65 to 77°C there was a significant decrease ($P < 0.05$) in the quantity of lecithin bound to dispersed protein, and this quantity at 77°C was significantly smaller ($P < 0.05$) than the quantity at 25°C. Heating the myoglobin emulsion from 25 to 65°C caused formation of a precipitate as described for heated sarcoplasmic protein-phospholipid emulsions. However, heating from 65 to 77°C did not affect any of the phospholipid classes in the bound or free lipid associated with precipitated protein.

Significant difference was not observed between quantities of free and bound lipid associated with total protein in heated

sarcoplasmic protein-phospholipid emulsions. However, when total protein was separated into dispersed and precipitated protein in the heated emulsions, a partitioning of the phospholipids was found. Significantly larger quantities of bound and free lipids were associated with precipitated protein than with dispersed protein ($P < 0.05$). The quantity of free lipid associated with dispersed protein was significantly smaller than any other type lipid association. When the bound and free lipids associated with dispersed or precipitated protein were separated into phospholipid classes, phospholipid classes were partitioned differently between bound and free lipid associated with dispersed or precipitated protein. Significantly larger quantities of sphingomyelin, lecithin and cephalin were associated with precipitated protein than with dispersed protein. Generally, significantly larger quantities of sphingomyelin and cephalin were in the free lipid than in bound lipid associated with the precipitated protein, but the lecithin quantities were distributed fairly evenly between bound and free lipid. Lysolecithin was preferentially found in the lipid bound to dispersed and precipitated protein, and ninhydrin-positive products were found only in lipid bound with dispersed protein.

The partitioning of the lipid (bound and free) and the phospholipid classes that was in heated sarcoplasmic protein-phospholipid emulsions also was seen generally in the heated myoglobin-phospholipid emulsions with one exception. Significantly larger quantities of cephalin associated with precipitated protein were in the bound lipid than in the free lipid.

As a rule significantly larger quantities of lecithin than of any other phospholipid class were in lipid (bound or free) associated with any state of protein (total, dispersed or precipitated).

CHAPTER V

GENERAL SUMMARY

Different steps in reported procedures of determining myoglobin concentration in aqueous extracts of bovine muscle (Rickansrud and Henrickson, 1967; Götze, 1969; Ledward, 1971), a proposed simplified procedure based on the reported procedures, and the possibility of using polyacrylamide disc gel electrophoresis as a quantitative tool to determine myoglobin concentration were investigated. Highest recoveries of myoglobin in solutions containing horse heart myoglobin or bovine myoglobin were obtained using the proposed simplified procedure for the determination of myoglobin concentration. This procedure involved: (1) the addition of a 4.5 M phosphate solution to a myoglobin-containing solution in ratio of 2:1 v/v, resulting in a 3 M phosphate solution containing only myoglobin and (2) the determination of the myoglobin concentration from absorbance at the isosbestic point of all forms of myoglobin, 525 nm. Efforts to use densitometric tracings of the polyacrylamide disc gel electrophoretic pattern of bovine sarcoplasmic proteins to determine myoglobin content in a solution containing such proteins were unsuccessful.

The simplified procedure for determination of myoglobin concentration was used in an investigation of possible interactions between phospholipids and bovine myoglobin, either isolated or as a component of sarcoplasmic proteins. Investigation of the possible interactions

was carried out as follows. Phospholipids and sarcoplasmic proteins were extracted from bovine ground round and myoglobin was prepared from sarcoplasmic protein extracts by ammonium sulfate fractionation. Solutions containing sarcoplasmic proteins (1.4 g proteins/100 g solution), sarcoplasmic protein-phospholipid emulsions (1.4 g protein and 0.8 g phospholipids/100 g emulsion) and phospholipid emulsions (0.8 g phospholipids/100 g emulsion) were heated from 25°C to end point temperatures of 56, 60, 65 and 77°C at a rate of temperature increase of 1°C/min. Solutions containing myoglobin (0.2 g protein/100 g solution) and myoglobin-phospholipid emulsions (0.2 g protein and 0.8 g phospholipid/100 g emulsion) were heated from 25°C to end point temperatures of 65 and 77°C at the same rate of temperature increase. The following measurements were made on protein dispersions at 25°C and all heated protein dispersions: (1) pH; (2) percent protein remaining dispersed at the pH of the dispersion and after pH of the dispersion that had been heated was adjusted to 7.00; (3) myoglobin concentration after isolation in 3 M phosphate solution; (4) available lysine, basic group equivalents and acidic group equivalents of dispersed and total protein in the dispersions and (5) densitometric tracings of the polyacrylamide disc gel electrophoretic patterns of dispersed proteins. The following measurements were conducted on protein-containing emulsions: (1) quantities of free and bound lipid associated with dispersed, precipitated and total protein in the emulsions; (2) quantities of phospholipid classes (lysolecithin, sphingomyelin, lecithin, cephalin, ninhydrin-positive products) in the free and bound lipid associated with

dispersed, precipitated and total protein in the emulsions; and (3) increase in water-soluble phosphorus which was used to determine decomposition of phospholipids to water soluble phosphorus compounds. The following measurements were conducted only on phospholipid emulsions: (1) lipid concentration, (2) phospholipid classes and (3) increase in water soluble phosphorus.

The investigation of heated bovine protein-phospholipid emulsions indicated that there were interactions between phospholipids and myoglobin either isolated or as a component of sarcoplasmic proteins and interactions between phospholipids and sarcoplasmic proteins. The interactions between phospholipids and sarcoplasmic proteins involved mainly weak polar bonds but interaction between phospholipids and isolated myoglobin involved both polar and possibly non-polar bonds as well as either covalent or strong complex bonds between phosopholipids and the basic groups especially the ϵ-NH_2 of lysine in the myoglobin.

Formation of a sarcoplasmic protein-phospholipid complex that was significantly more stable ($P < 0.05$) to heat precipitation than sarcoplasmic proteins at the pH range of 5.30 to 5.75 was indicated by the values for percent protein remaining dispersed in heated dispersions. Formation of a myoglobin-phospholipid complex that was significantly less stable ($P < 0.05$) to heat precipitation than myoglobin also was indicated. Both of the complexes after heating were less stable to a higher pH (7.00) than the heated proteins.

The determination of myoglobin concentration by isolation in 3 M phosphate solutions in heated sarcoplasmic protein dispersions indicated

the formation of a myoglobin-phospholipid-protein complex. This complex was significantly less stable to heat precipitation ($P < 0.05$) than a myoglobin-protein complex at 65°C but significantly more stable ($P < 0.05$) to heat precipitation at 77°C.

The densitometric tracings of the electrophoretic pattern of bovine sarcoplasmic proteins in dispersions containing the proteins (solutions and emulsions) at 25°C and at each end point temperature showed that: (1) the electrophoretic pattern of myoglobin was altered by mechanical blending of protein-containing solutions but not by blending of the emulsions; (2) heating both dispersions to 60°C caused the protein peaks which probably represented glycolytic enzymes to disappear completely; (3) heating both dispersions to 65°C caused the protein peak which probably represented a myoalbumin to disappear; (4) myoglobin peaks in the emulsion and in the protein-containing solution were still present in dispersions heated to 77°C, but the peaks were smaller than at lower temperatures; and (5) the myoglobin peak in emulsion heated to 77°C appeared to be larger than the myoglobin peak in the protein-containing solution heated to 77°C.

Many other interesting phenomena were observed in the myoglobin-phospholipid emulsions. At 25°C there apparently were interactions between phospholipids and myoglobin that caused: (1) abnormally high values for acidic group equivalents per mole of myoglobin; (2) significantly lower pH values in the emulsion than in the myoglobin-containing solution ($P < 0.05$); (3) precipitation of myoglobin in 3 M phosphate solutions, pH 6.6; and (4) failure of myoglobin to migrate in the

polyacrylamide disc gel electrophoresis at a pH of 8.1. Heating the myoglobin-phospholipid emulsions from 25 to 65 and 77°C caused: (1) the acidic group equivalents of myoglobin to return below the total number of acidic group residues per mole myoglobin; (2) the pH of the emulsion to increase significantly ($P < 0.05$); (3) the solubility of myoglobin in 3 M phosphate solution to increase significantly ($P < 0.05$); (4) the amount of myoglobin migrating in gels at pH 8.1 to increase greatly; and (5) the number of ϵ-NH_2 groups of lysine in the myoglobin to increase significantly ($P < 0.05$).

Heating the phospholipid emulsion, myoglobin-phospholipid emulsion, and the sarcoplasmic protein-phospholipid emulsion to 77°C caused (1) significant increases ($P < 0.05$) in water-soluble phosphorus in phospholipid emulsions and in myoglobin-phospholipid emulsions which showed that approximately 1 percent of the total phospholipids in the emulsions decomposed to water-soluble phosphorus compounds and (2) significant increases ($P < 0.05$) in ninhydrin-positive products tentatively identified as lysocephalin and a geometric isomer of cephalin in sarcoplasmic protein-phospholipid emulsions which showed that approximately 10 percent of the total phospholipids in the emulsion was altered to these products.

Heating the sarcoplasmic protein-phospholipid emulsions generally: (1) decreased significantly ($P < 0.05$) the free lipid (unbound and hydrophobically bound) associated with dispersed protein; (2) decreased significantly ($P < 0.05$) the quantity of lecithin in free or bound lipid associated with dispersed protein; (3) decreased

significantly ($P < 0.05$) the quantity of cephalin in free lipid associated with dispersed protein; (4) increased significantly ($P < 0.05$) the quantities of lysolecithin and ninhydrin-positive products of lipid bound to dispersed protein. Heating the emulsion from 25 to 56°C caused the formation of a precipitate containing protein associated with free and bound lipid in which all phospholipid classes were represented.

Heating the myoglobin-phospholipid emulsions: (1) from 25 to 65°C caused a significant increase ($P < 0.05$) in the amount of lecithin in free and bound lipid associated with total or dispersed protein and (2) from 65 to 77°C caused a significant decrease ($P < 0.05$) in the quantity of lecithin in lipid bound to dispersed protein, and this quantity at 77°C was significantly smaller than the quantity at 25°C. Heating the myoglobin-phospholipid emulsion from 25 to 65°C caused formation of a precipitate as described for heated sarcoplasmic protein-phospholipid emulsions.

When the total protein of heated sarcoplasmic protein-phospholipid emulsions was separated into dispersed and precipitated protein, a partitioning of the phospholipids was found. Significantly larger quantities of bound and free lipids were associated with precipitated protein than with dispersed protein at the $P < 0.05$ level. The quantity of free lipid associated with dispersed protein was significantly smaller than any other type lipid association. When the bound and free lipids associated with dispersed or precipitated protein were separated into phospholipid classes, phospholipid classes were partitioned differently between bound and free lipid associated with

dispersed or precipitated protein. Significantly larger quantities of sphingomyelin, lecithin and cephalin were found in bound and free lipid associated with precipitated protein than with dispersed protein ($P < 0.05$). Generally, significantly larger quantities of sphingomyelin and cephalin were found in the free lipid than in bound lipid associated with the precipitated protein. However, the lecithin quantities were distributed fairly evenly between the bound and free lipid. Significantly larger quantities of lysolecithin were found in bound lipid than in free lipid of either dispersed or precipitated protein, but ninhydrin-positive products were found only in lipid bound to dispersed protein.

The partitioning of the lipid and the phospholipid classes in heated sarcoplasmic protein-phospholipid emulsions was found generally to be the same in heated myoglobin-phospholipid emulsions with one exception. Significantly larger quantities of cephalin associated with precipitated protein were found in the bound lipid than in the free lipid.

In heated emulsions there were interactions between phospholipids and myoglobin, either isolated or as a component of sarcoplasmic proteins, that affected the heat precipitation of myoglobin. Interactions of phospholipid with myoglobin alone destabilized the myoglobin to heat precipitation. Interactions with myoglobin as a component of sarcoplasmic proteins destabilized the myoglobin to heat precipitation at 65°C but protected myoglobin somewhat from heat precipitation at 77°C. Phospholipid interactions with proteins do affect myoglobin precipitation at temperatures to which meat is normally

cooked and these interactions could affect directly the color of cooked meat since the color of cooked meat depends largely upon the nature and amount of myoglobin derivatives present.

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APPENDICES

APPENDIX A

LIPID RECOVERY IN PHOSPHOLIPID EMULSIONS

The quantities of lipid for all samples in an emulsion and the quantity lipid recovered for that emulsion by washing were summed. The total weight lipid recovered and the percent recovery for each phospholipid emulsion that was investigated are shown below.

TABLE 31

THE WEIGHT OF LIPID RECOVERED AND PERCENT RECOVERY IN PHOSPHOLIPID EMULSIONS

Final Temperature of Sample, °C	Emulsion 1	Emulsion 2
	----weight lipid, g--------	
25	0.1440	0.1202
56	0.1444	0.1564
60	0.1983	0.1495
65	0.1261	0.1213
77	0.1515	0.1639
Total weight in samples	0.7633	0.7113
Weight lipid from washing	0.0270	0.0148
Total weight lipid recovered	0.7903	0.7261
Percent recovery	96.2	89.3

$$\text{Percent recovery} = \frac{\text{total weight lipid recovered}}{\text{weight lipid in emulsion before heating}} \times 100 .$$

APPENDIX B

RECOVERY OF PHOSPHOLIPIDS IN HEATED PROTEIN-LIPID EMULSIONS

Example of Calculation

Sarcoplasmic Protein-Phospholipid Emulsion Heated to 60°C

Source of lipid	Grams
1. Weight lipid in sample of emulsion before heating for determination of lipid concentration	0.0394
2. Weight lipid in supernatant (II)[1] of heated emulsion after centrifugation and reemulsification	0.2309
3. Weight lipid in centrifugate I[1] divided into samples for measurements (III) and for lyophilization (IV)	0.3474
4. Weight lipid recovered in washing procedure	0.1505
Total weight lipid regained	0.7682

$$\text{Percent recovery} = \frac{\text{total weight lipid regained}}{\text{weight lipid added}} \times 100 = \frac{0.7682}{0.8559} \times 100$$

$$= 89.7 .$$

[1] Figure 6, page 43. The weight lipid in source 2 was calculated by determining lipid concentration (bound and free) in sample that was lyophilized and then multiplying the weight of supernatant II by lipid concentration. The weight lipid in source 3 was calculated by determining lipid concentration (bound and free) and multiplying the weight of slurried centrifugate I by that lipid concentration.

APPENDIX C

LIPID RECOVERY IN PROTEIN-LIPID EMULSION

TABLE 32

PERCENT LIPID RECOVERY IN HEATED PROTEIN-PHOSPHOLIPID EMULSIONS

Emulsion	Sample[a]	Final Temperature[b] °C	Percent Recovery
Sarcoplasmic protein	1	25	--
Sarcoplasmic protein	2	25	--
Sarcoplasmic protein	1	56	101.4
Sarcoplasmic protein	2	56	101.0
Sarcoplasmic protein	1	60	94.7
Sarcoplasmic protein	2	60	89.8
Sarcoplasmic protein	1	65	99.2
Sarcoplasmic protein	2	65	96.3
Sarcoplasmic protein	1	77	98.9
Sarcoplasmic protein	2	77	96.9
Myoglobin	1	25	--
Myoglobin	2	25	--
Myoglobin	1	65	88.5
Myoglobin	2	65	91.5
Myoglobin	1	77	84.0
Myoglobin	2	77	84.8

[a]Sample number 1 represents the first emulsion prepared and heated to a given end-point temperature (except for 25°C) and number 2 represents a duplicate emulsion treated in the same way.

[b]Final temperature is the 25°C or the end point temperature of the emulsion.

APPENDIX D

PERCENT PHOSPHOLIPID CLASSES IN EMULSIONS

TABLE 33

PERCENT PHOSPHOLIPID CLASSES IN HEATED PHOSPHOLIPID EMULSIONS

Lipid Class	Temperatures to Which Emulsions Were Heated				
	25°C	56°C	60°C	65°C	77°C
	% lipid class				
Lysolecithin	4.86	7.28	6.60	5.74	8.27
Sphingomyelin	8.93	9.46	9.98	8.75	9.79
Lecithin	52.10	49.87	51.05	53.00	52.48
Cephalin	34.10	33.40	32.37	32.50	29.46

TABLE 34

PERCENT PHOSPHOLIPID CLASSES IN BOUND AND FREE LIPID OF HEATED SARCOPLASMIC PROTEIN-PHOSPHOLIPID EMULSIONS

Lipid Class Protein State Lipid Association	Temperature to Which Emulsions Were Heated 25°C	56°C	60°C	65°C	77°C
	% lipid class				
Lysolecithin					
Dispersed protein					
Bound lipid[d]	8.12	8.47	13.76	10.30	16.91
Free lipid[e]	2.95	3.47	5.25	3.59	4.65
Precipitated protein					
Bound lipid[d]	--	7.14	7.72	6.12	10.52
Free lipid[e]	--	3.47	3.11	2.00	4.43
Sphingomyelin					
Dispersed protein					
Bound lipid[f]	7.38	4.47	5.50	4.56	4.52
Free lipid[g]	11.77	14.75	6.73	12.86	--
Precipitated protein					
Bound lipid[f]	--	7.53	8.15	8.15	9.02
Free lipid[g]	--	14.75	11.76	12.58	15.99
Lecithin					
Dispersed protein					
Bound lipid[d]	65.00[c]	49.15[b,c]	47.77[b,c]	50.37[b]	28.52[a]
Free lipid[e]	55.04	61.82	77.44	52.69	57.37
Precipitated protein					
Bound lipid[d]	--	70.16	64.25	64.93	61.89
Free lipid[e]	--	61.82	79.67	53.76	55.26
Cephalin					
Dispersed protein					
Bound lipid[f]	18.60	17.70	8.65	9.58	13.46
Free lipid[g]	30.26	27.35	10.60	30.86	37.98
Precipitated protein					
Bound lipid[f]	--	15.17	10.61	20.81	18.58
Free lipid[g]	--	27.35[a,b]	32.02[a,b]	31.66[b]	24.33[a]
Ninhydrin-positive Products					
Dispersed protein					
Bound lipid	0.91[a]	20.22[a,b]	24.33[a,b]	25.20[a,b]	36.59[b]

[a,b,c]Percent lipid classes in the same row bearing like superscript letters are not significantly different ($P < 0.05$).

[d,e]Bound lipid has a significantly greater percentage ($P < 0.01$) of the lipid class than has the free lipid.

[f,g]Bound lipid has a significantly smaller percentage ($P < 0.01$) of the lipid class than has the free lipid.

TABLE 35

PERCENT PHOSPHOLIPID CLASSES IN BOUND AND FREE LIPID OF HEATED MYOGLOBIN-PHOSPHOLIPID EMULSIONS

Lipid Class Protein State Lipid Association	Temperatures to Which Emulsions Were Heated 25°C	65°C	77°C
	% lipid class		
Lysolecithin			
Dispersed protein			
Bound lipid	9.70	8.26	19.37
Free lipid	5.00	4.03	4.74
Precipitated protein			
Bound lipid	--	7.86	5.49
Free lipid	--	7.30	5.90
Sphingomyelin			
Dispersed protein			
Bound lipid[a]	5.49	9.69	12.40
Free lipid[b]	14.60	14.61	13.81
Precipitated protein			
Bound lipid[a]	--	11.56	8.20
Free lipid[b]	--	16.35	17.84
Lecithin			
Dispersed protein			
Bound lipid	67.71	63.58	49.66
Free lipid	66.06	70.42	70.51
Precipitated protein			
Bound lipid	--	65.37	70.21
Free lipid	--	59.13	62.97
Cephalin			
Dispersed protein			
Bound lipid	13.90	12.70	18.58
Free lipid	14.35	10.95	10.96
Precipitated protein			
Bound lipid	--	15.21	16.10
Free lipid	--	17.23	13.28
Ninhydrin-positive Products			
Dispersed protein			
Bound lipid	3.43	5.79	--

[a,b]Free lipid has a significantly greater percentage of sphingomyelin than has the bound lipid ($P < 0.05$).

VITA

Sharon Lynn Melton, daughter of Robert and Fleda M. Taylor, was born January 1, 1940, at Paris, Arkansas. She attended elementary and secondary schools at Ozark, Arkansas, and graduated from high school in May 1956. She attended Arkansas Polytechnic College from September 1956 to June 1958, and transferred to the University of Arkansas in September 1958. At the University of Arkansas she majored in chemistry and received the Bachelor of Science degree in June 1960, and the Master of Science degree in January 1963. She was employed in various research laboratories at the University of Arkansas and Kansas State University from 1961 to 1970 except for five months in 1962 when she worked at Reichold Chemicals, Tacoma, Washington. She began study toward the Ph. D. degree in Food Science at The University of Tennessee, Knoxville, in the fall of 1970.

She received an undergraduate NSF research grant in 1959 and the award for top graduating chemist at the University of Arkansas in 1960. She also received graduate research assistantships from the University of Arkansas in 1960 and from The University of Tennessee in 1970. She was initiated into Omicron Nu and Sigma Xi at The University of Tennessee, and she is a member of the American Chemical Society and the Institute of Food Technologists.

She married Curtis C. Melton, January 23, 1958, and has one son, Dennis Coy, born January 24, 1967.