PORK MUSCLE PROTEIN

Ph.D. Agricultural Chemistry

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ABSTRACT

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Attempts were made to isolate proteins from water extracts of pork skeletal muscle by a number of techniques which involved solvent and salt precipitations, column chromatography and preparative electrophoresis. A sample of myoglobin was prepared which was homogeneous as measured by starch-gel electrophoresis and by ultra-centrifugation. Crystalline myoglobin was prepared from the homogeneous myoglobin. Ultracentrifugal, amino acid and other analyses showed that pork myoglobin has a sedimentation coefficient of 1.95 S, a molecular weight of 17,500 (M_b) and 18,400 (M_m), contains 151 amino acid residues and has valine as the amino-terminal amino acid. Amino acid analyses of carboxypeptidase digest of pork myoglobin suggest that serine is the carboxy-terminal amino acid.

PORK MUSCLE PROTEIN

A THESIS

by

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GENERAL INTRODUCTION

Myoglobin and hemoglobin are among the proteins that have been studied in great detail. This is because of their importance in animal physiology and because of the role which they play in the curing of meat.

Myoglobin is an intra-cellular pigment which is distributed among invertebrates and vertebrates. It occurs in the latter in red muscle. Myoglobin is involved in the mechanism of oxygen storage rather than oxygen transport in the living organism (Wyman, 1948).

Despite the extensive work that has been carried out on the myoglobins from a number of species, there is little information available on the myoglobin from pork muscle. It is unlikely that the physico-chemical properties of different myoglobins are identical.

Several workers (Morgan, 1936; Roche et al., 1942; Lewis and Schweigert, 1955) were unsuccessful in their attempts to crystalize pork myoglobin. Morgan (1936) and Kendrew et al. (1954) pointed out that the difference in the ease of crystallization of myoglobins from different species of animal suggested that there is species difference in the myoglobins.

The object of this study was (a) to devise a method for the isolation of proteins and in particular myoglobin, from water soluble extracts of pork muscle and (b) to gain information on the constitution of the isolated proteins and their physical properties.

I. HISTORICAL REVIEW

It is difficult to separate the studies that have been made on myoglobins from those made on hemoglobins. Gunther (1921) proposed the name myoglobin when he became convinced that the muscle and the blood pigments were different.

The distinction between the myoglobins and hemoglobins dates back to the work of MacMunn (1881). But the existence of a protein in muscle that resembled blood hemoglobin remained in doubt until Theorell (1932) crystallized myoglobin from horse heart. Theorell's work on the isolation of a crystalline material and his studies on its properties (1934a,b,c,d) stimulated research on the myoglobins.

Theorell (1934a) who determined the sedimentation coefficient of myoglobin reported that there were two components of myoglobin in horse heart and that most of the protein components had molecular weights of 34,000 but others had molecular weights of 68,000. Later, Svedberg (1937) re-examined the results of Theorell (1934a) and found the molecular weight of myoglobin to be about 17,500. Polson (1939) confirmed the results of Svedberg.

The most widely used method of isolation of

myoglobins from animal tissue is basically that of Theorell (1932) which involves ammonium sulfate fractionation. Morgan (1936) studied the solubility of myoglobin in concentrated ammonium sulfate and phosphate solutions, and demonstrated a great difference in solubility between myoglobin and hemoglobin. Drabkin (1950) modified Theorell's method of isolation of myoglobin by taking advantage of the solubility difference between myoglobin and hemoglobin in concentrated salt solution.

Theorell and de Duve (1947) first showed that crystalline myoglobin (human) was not electrophoretically (Tiselius) homogeneous. Schmid (1949) separated crystalline whale myoglobin into two or three electrophoretic components. Lewis (1954) separated crystalline horse myoglobin into three electrophoretic components. The horse myoglobin appeared to be homogeneous when subjected to ultra-centrifugation.

Boardman and Adair (1956) separated horse myoglobin into two fractions by column chromatography using Amberlite IR-50 resin. Later seal (Rumen, 1959), sperm whale (Edmundson and Hirs, 1961), human (Perkoff et al., 1962), finback whale (Atassi and Saplin, 1966), harbor seal and porpoise (Hapner et al., 1968) myoglobins were each separated chromatographically into five fractions by the use of

CM-cellulose, Amberlite IR-50 resin, DEAE-cellulose, CM-cellulose and CM-Sephadex respectively. The authors demonstrated that each of the components that were isolated was electrophoretically homogeneous. The amino acid analyses of many workers (Akeson and Theorell, 1960; Rumen, 1960; Edmundson and Hirs, 1962a; Perkoff et al., 1962; Atassi and Saplin, 1966) have shown that despite the differences in the electrophoretic mobility of the different components of the myoglobin from a given species, the components had identical amino acid composition.

Perkoff et al. (1962) suggested that the differences in chromatographic and electrophoretic behavior of the myoglobin components were the result of the differences in the state of the iron in the heme prosthetic group. Atassi and Saplin (1966) separated finback myoglobin into a number of fractions by means of column chromatography. Electrophoretic analysis of the fractions showed that they contained one or more electrophoretic components. The authors demonstrated that subjection of a given fraction to physical treatment such as dialysis against distilled water and freeze drying resulted in a change in the number of electrophoretic components in the chromatographically separated fractions. The authors suggested that the heterogeneity

of crystalline myoglobin might be the result of conformational change in the protein molecule.

1. PROSTHETIC GROUP

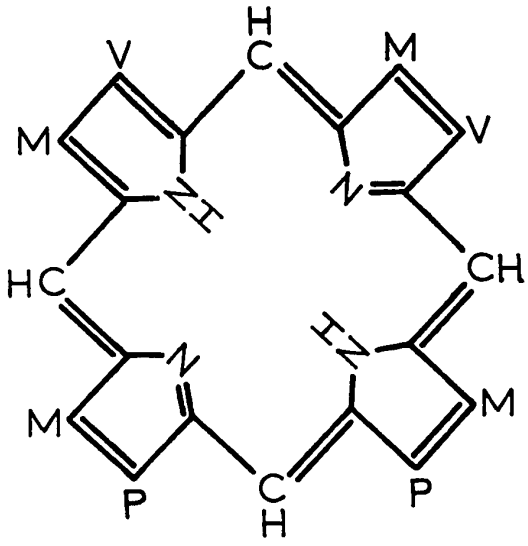
Schönheimer (1929) showed that the prosthetic group of myoglobin was a type IX protohematin. Figure 1 shows the structure of protoporphyrin IX.

The coordination linkage of the iron in the heme proteins may be ionic, covalent or of intermediate character, the type of linkage being reflected in the magnetic properties of the substances (Lemberg and Legge, 1949). It has been generally accepted that the iron atom in a heme molecule is able to combine only with two other groups. The introduction of two simple nitrogenous molecules into a ferroprotoporphyrin (heme) gives rise to a substance known as a hemochromogen or a hemochrome.

The modern chemistry of the ligand complexes of heme compounds began about 1925, when Anson and Mirsky (1925) distinguished between hemochromogen and heme. They recognized that heme was the true isolated prosthetic group of hemoglobin.

Heme can combine with a great variety of nitrogenous substances to form hemochromogens. The nitrogenous compounds include: ammonia, primary amines, carbylamines,

Figure 1. The Structure of Protoporphyrin IX



M: $-CH_3$

V: $-CH=CH_2$

P: $-CH_2CH_2CO_2H$

Protoporphyrin IX	$C_{20}H_6N_4(CH_3)_4(C_2H_3)_2(CH_2CH_2CO_2H)_2$
Mesoporphyrin IX	$C_{20}H_6N_4(CH_3)_4(C_2H_5)_2(CH_2CH_2CO_2H)_2$
Deuteroporphyrin IX	$C_{20}H_6N_4(CH_3)_4(H)_2(CH_2CH_2CO_2H)_2$
Hematoporphyrin IX	$C_{20}H_6N_4(CH_3)_4(CHOHCH_3)_2(CH_2CH_2CO_2H)_2$

hydrazine, pyridine, and pyridine compounds such as nicotine, imidazole compounds, piperidine and nitric oxide. Heme can also combine with denatured proteins and cyanides (Lemberg and Legge, 1949).

Free heme in aqueous solutions was found to be dimeric, and each iron atom of the dimer was coordinated to a water molecule (Shack and Clark, 1947).

In hematin (ferric protoporphyrin or ferric heme) the iron is in the ferric state rather than in the ferrous state as in the heme. Hematin also combines with the ligands described previously for heme to form hemochromogens (Lemberg and Legge, 1949).

Shack and Clark (1947) found that hematin was polydispersed in aqueous solutions. They reported that the particle weight of the hematin ranged from 3,000 to 65,000. Inada and Shibata (1962) attributed this high particle weight of hematin in aqueous solutions to the micellar structure formed by the aggregation of the monomeric or dimeric units.

2. GLOBIN

Bertin-Sans and de Moitessier (1893) demonstrated that hemoglobin could be split at low pH into heme and globin. The authors claimed the resynthesis of oxyhemoglobin

from heme and globin. Similar observations were made later by Anson and Mirsky (1925). In 1926, Hill and Holden (1926) prepared globin from hemoglobin which was soluble at neutral pH. The authors also reported that on combination with hematin the globin gave a material which had spectroscopic properties that were very similar to those of the original hemoglobin. Anson and Mirsky (1930) prepared globin from hemoglobin, which contained a high proportion of water soluble protein. This method like that of Hill and Holden (1926), was based on the treatment of an aqueous solution of hemoglobin with dilute hydrochloric acid (0°C) and subsequent precipitation of globin with cold acetone. The protein hydrochloride so obtained was slowly neutralized by the addition of sodium hydroxide and the insoluble material was removed. Anson and Mirsky (1930) showed that the preparation of globin by their method involved the denaturation and the subsequent renaturation of the protein.

Drabkin (1945) prepared denatured globin from crystalline horse myoglobin and succeeded in renaturing it completely. He also claimed to have coupled the globin with heme in 100% yield. The resultant synthetic myoglobin had the same crystalline structure as the original

protein. Theorell and Akeson (1955) prepared native globin from homogeneous horse myoglobin. The globin obtained was homogeneous in the ultracentrifuge at neutral pH. The globin had a sedimentation coefficient of 1.97S and a molecular weight of about 18,000. These authors showed that the myoglobin prepared from this globin had the same absorption spectrum (220 mμ to 700 mμ), sedimentation coefficient, electrophoretic behavior and crystal forms as did the original myoglobin.

Rumen and Appella (1962) prepared globin from seal myoglobin and showed that at pH 3 to pH 5 the globin aggregates to form components with sedimentation coefficients of about 4.5 and 8S.

Rossi-Fanelli et al. (1964) stated that the dependence of the sedimentation coefficient of myoglobin on concentration under normal conditions (neutral pH, moderate ionic strength (μ = 0.1) and temperature near 20°C) is small. They stated that the value of the sedimentation coefficient at 1% concentration is about 5% lower than the value obtained by extrapolation to zero protein concentration.

Rossi-Fanelli et al. (1958c) reported that the myoglobins of the marine molusks Aplysis depliance, and

limacina, were very different in amino acid composition from that of mammalian myoglobins but had the same iron content, molecular weight, and sedimentation coefficient.

The amino acid composition of myoglobins has been determined by many workers (Timmer et al., 1957; Konosu et al., 1958; Rossi-Fanelli et al., 1958c; Akeson and Theorell, 1960; Rumen, 1960; Edmundson and Hirs, 1961; Perkoff et al., 1962; Hirs and Olcott, 1964; Karadzova et al., 1964; Atassi and Saplin, 1966; Hapner et al., 1968). The authors showed that various mammalian myoglobins had very similar amino acid compositions. It was noted that myoglobins isolated from fish (e.g. blue fin tuna (Konosu et al., 1958), albacore tuna, shipjack tuna, and chinook salmon (Brown et al., 1962) and yellow fin tuna (Hirs and Olcott, 1964)) all contained cysteine, while the mammalian myoglobins did not contain cysteine. Atassi (1966) reviewed the available literature on the amino acid composition of myoglobins and hemoglobins. He reported that the amino acid composition of fish myoglobins resembled more closely the amino acid composition of mammalian hemoglobins than they did mammalian myoglobins.

The chemical investigation of Edmundson and Hirs (1961), together with X-ray crystallographic studies of

Kendrew et al. (1960, 1961) made possible the assignment of most of the amino acid residues to the three dimensional structure of sperm whale myoglobin. Edmundson and Hirs (1962a,b,c) and Edmundson (1965) worked out the complete amino acid sequence of sperm whale myoglobin.

3. MOLECULAR STRUCTURE

Hill and Holden (1926) reported that metalloporphyrins in which the iron was replaced by another element (Mn, Co, Ni, Cu, Zn, Sn) were able to combine with globins, but such metalloporphyrin globin compounds were not able to unite with oxygen reversibly.

It is well known that heme combines with the proteins, apo-myoglobin and apo-hemoglobin, to yield myoglobin and hemoglobin respectively. Myoglobin and hemoglobin combine with oxygen to form compounds that can dissociate to yield oxygen. The iron in the oxygen complex remains in the ferrous state. This unique property distinguishes myoglobin and hemoglobin from other classes of hematin compounds (Lemberg and Legge, 1949). Lemberg and Legge (1949) pointed out that the reversible combination of myoglobin and hemoglobin is possible because of the stability of the ferrous-oxygen complex. They also pointed out that the difference between the stability of oxy-myoglobin and

oxy-hemoglobin from that of other oxy-hematin compounds is a matter of degree of stability, as even globin is unable to prevent the slow oxidation of the ferrous ion in myoglobin and hemoglobin.

Wang et al. (1958) pointed out that the combination of heme with globins resulted in the protection of the iron in the heme from autoxidation. They suggested that the stability of oxy-myoglobin and oxy-hemoglobin is due to the fact that the heme part of the molecule is surrounded by lyophobic groups of the peptide chain. They reasoned that under these conditions where the heme is surrounded by groups of low polarity, the autoxidation of the iron would be slow.

Perutz et al. (1960) and Kendrew et al. (1961) showed by means of X-ray crystallographic studies that the regions of the peptide chain which surround the heme moiety is rich in aromatic amino acid residues.

Lumry (1961) suggested that the protein exerts a stabilizing effect on the iron-oxygen complex by steric hindrance.

Antonini et al. (1961) showed that hemo-proteins differ markedly in their affinity for oxygen and resistance to autoxidation of their oxy-compounds. The authors

demonstrated that myoglobin has a higher affinity for oxygen than does hemoglobin and that the ferrous ion in oxymyoglobin is more readily oxidized than it is in oxyhemoglobin. When hemoglobin was modified by treatment with carboxypeptidase A and B, the resultant product had a higher oxygen affinity than did myoglobin, but the ferrous ion in the modified hemoglobin was less readily oxidized than it was in myoglobin.

St. George and Pauling (1951) showed that the magnitude of the equilibrium constant between hemoglobin and isocyanides were in order of, ethyl isocyanide>isopropyl isocyanide>t-butyl isocyanide. The authors proposed a hypothesis of "buried hemes" to explain the characteristic sigmoid nature of the equilibrium of hemoglobin with gases such as oxygen or carbon monoxide. The hypothesis of buried heme did not appear to be applicable to the equilibrium of myoglobin and oxygen as the characteristic equilibrium curve was hyperbolic in shape rather than sigmoid in shape. Lein and Pauling (1956) showed that the equilibrium constants between myoglobin and isocyanides, however, were in the same order as they were for hemoglobin. They concluded that the hypothesis of "buried hemes" was applicable even though the molecular weight of myoglobin is only

one fourth of that of hemoglobin.

Later, Kendrew et al. (1961) and Cullis et al. (1962) located the position of the heme in myoglobin and in hemoglobin by X-ray crystallographic measurements. Their results showed conclusively that the heme moiety was not buried in the interior of the molecule.

Kendrew et al. (1958) and Bodo et al. (1959) studied the structure of metmyoglobin by means of X-ray diffraction measurements (6 Å resolution). They showed that one polypeptide chain of metmyoglobin was a rodlike structure, roughly circular in cross section and was made up of straight segments joined to each other by irregular regions which formed the corners of a constructed model. The heme group was situated in a pocket formed by a loop in the polypeptide chain. However, the heme moiety appeared to be near the surface of the three dimensional molecular model. The heme group appeared to be in close contact with several segments of the peptide chain.

Kendrew et al. (1960, 1961) constructed a three dimensional model of a metmyoglobin molecule by means of X-ray diffraction data (2 Å resolution) and assigned positions for the amino acid residues in the model with the aid of the results of amino acid composition analysis

(Edmundson and Hirs, 1961). They observed that approximately 70% of the chain had a right-handed α-helix configuration and that there were eight helical regions which made up the straight segments (A-H). There were also eight non-helical regions, seven interposed between helical regions and one at the carboxyl end of the chain. All four proline residues that were present in the molecule corresponded to the corners in the peptide chain. They also noted that the polar side chains of the amino acid residues were mainly directed toward the outside of the molecule, and the non-polar side chains toward the inside. They noted that the polar groups interacted with the corresponding polar groups of the chain or with the solvent. The authors also noted that the non-polar interactions between the lyophobic amino acid residues were greater than the polar interactions. They suggested that this is of great importance in the stabilization of the myoglobin molecule. They observed that the structure of myoglobin was very compact and that there seemed to be no space for liquid inside the protein structure.

Drabkin (1945) and Theorell and Akeson (1955) showed that the physico-chemical properties (crystal forms, sedimentation coefficient, molecular weight, and absorption spectrum 220 mμ to 700 mμ) of reconstituted myoglobins

were similar to those of native myoglobins. Rossi-Fanelli et al. (1958b) reported that the coupling capacity of globin for hematin was stoichiometric (4 moles heme per mole of reconstituted hemoglobin). Rossi-Fanelli and Antonini (1959a,b) and Antonini and Gibson (1960) found that the oxygen equilibrium curve and the kinetics of the reactions of reconstituted hemoglobins were similar to those of natural hemoglobins. The authors concluded that the heme-binding sites in the globins must be very specific.

Coryell and Pauling (1940) studied the acidity of groups associated with the heme of hemoglobin and deduced that the iron-binding group in hemoglobin was the imidazole nitrogen of a histidine residue in the peptide chain. Kendrew et al. (1961) and Cullis et al. (1962) used X-ray diffraction data to show that one of the coordination bonds of the iron atom in the heme moiety of myoglobin and hemoglobin was directed toward the imidazole nitrogen of a histidine residue. The other coordination bond of the iron atom was attached to a water molecule or to a ligand.

Rossi-Fanelli and Antonini (1960) reported that the heme moiety in myoglobin, hemoglobin and in other hemeproteins is not a fixed or an immobile prosthetic group even at neutral pH. They observed that heme could dissociate

from the protein molecule and that the heme-globin interaction was a true equilibrium. The reversibility of the interaction between heme and globin was demonstrated by noting the transfer of the heme from one protein to another. They found that myoglobin had greater affinity for heme than did hemoglobin at neutral pH. Banerjee (1962) confirmed these observations.

Kendrew et al. (1961) suggested that the carboxyl groups of the propionic acid side chains of the porphyrin were of importance in linking porphyrin to the globin molecule. The authors constructed a model of myoglobin from the X-ray diffraction measurements and noticed that one of the carboxyl groups of the porphyrin was directed to an arginine residue in the peptide chain while the other one was directed toward the outside of the molecule. The vinyl side chains in the porphyrin were also found to be involved in the heme-globin linkage. In the X-ray model of myoglobin the vinyl groups of heme were directed toward the interior of the peptide chain. They noted that the heme rested in a pocket made by a loop in the peptide chain where it was surrounded almost entirely by non-polar residues.

Rossi-Fanelli and Antonini (1960) and Gibson and

Antonini (1963) found that meso-, deutero-, and hemato-hemes (see Fig. 1) all had much lower affinity toward globin than did proto hematin. These hematins were easily displaced from combination with the protein when proto hematin was added.

Stryer et al. (1964) suggested that the pyrrole rings or vinyl groups of the heme mostly was parallel to two aromatic rings in the peptide chain. The authors pointed out that the π-bonding interactions must play an important role.

Cann (1964, 1965, 1967a,b) studied the effect of aromatic residues on the stability of myoglobin by studying the kinetics of the reaction of sperm whale myoglobin with zinc and the effect of aromatic compounds (e.g. benzene) on urea denaturation of myoglobin. He found that the addition of benzene to the system enhanced the rate of reaction between myoglobin and zinc and the rate of urea denaturation of myoglobin. The author concluded that aromatic compounds disrupt π-bonding interactions between the two aromatic rings of the phenylalanine residues in the peptide chain and the heme moiety by complexing directly with the heme.

Scouloudi (1960) made X-ray measurements on seal

myoglobin and compared the results with those made by the Kendrew group on sperm whale myoglobin. The author found that in spite of the differences in their amino acid compositions the structures of the myoglobins were essentially the same.

II. METHODS

1. MOISTURE, ASH AND NITROGEN

Moisture, ash and nitrogen were determined by the methods described on page 346 and page 744 of the Methods of Analysis AOAC (1965).

2. SPECTRAL ANALYSIS

The ultraviolet and visible spectral analyses were made using a Spectronic Model 505 spectrophotometer.

3. STARCH-GEL ELECTROPHORESIS

The method of electrophoresis was essentially that described by Smithies (1955, 1959a,b).

Hydrolyzed starch (Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario) (36 g) was added to 60 ml of a suitable buffer solution contained in a suction flask (IL). Two hundred and forty milliliters of the required amount (300 ml) of buffer was heated to boiling and was added to the starch suspension contained in the suction flask. The flask was swirled vigorously while the buffer was being added to ensure thorough mixing. The mixture was then heated to boiling. The suction flask was connected to a water aspirator and a

negative pressure was applied in order to remove air bubbles from the solution. The starch solution was transferred to a gel forming tray, care being taken to avoid the trapping of air bubbles in the gel. The cover plate was placed in position and lead weights (ca. 800 g each) were placed on the cover at the corners. The gel was allowed to cool for five hours.

The procedure that was followed for the application of the protein solution to the gel for horizontal electrophoresis was different from that followed for vertical electrophoresis. For horizontal electrophoresis small pieces of filter paper which had been dipped in the protein solution, were placed in the slit that had been cut in the gel. The surface of the gel was covered with Saranwrap to seal the sample in the starch gel and to prevent the loss of moisture from the gel during electrophoresis. For vertical electrophoresis, the protein solution was introduced into the slots by means of a Pasteur pipette, and the surface of the gel was covered with Petrolatum N.F. The gel plate was mounted in position in the electrode compartment and several sheets of chromatographic paper (3MM) were used as a wick to connect the plate to the bridge solution. A voltage gradient of 12 volt/cm

(i.e. 350 volts across the gel for horizontal), or 7 volt/cm (i.e. 200 volts across the gel for vertical) was applied for a period of five hours (horizontal electrophoresis), or sixteen hours (vertical electrophoresis) in a cold room ($4^{o}C$). At the end of the run the Saranwrap, or Petrolatum N.F. was removed from the plate, and the starch-gel plate was transferred to a slicing tray, and the gel (0.6 cm thick) was sliced into two pieces (0.3 cm thick). The starch plate was stained for protein by means of a saturated amido black 10B solution (Methanol-Acetic acid-Water 5:1:5 by volume). The stained gel was then thoroughly washed with a mixture of Methanol-Acetic acid-Water (5:1:5 by volume). The washed electropherogram was wrapped in Saranwrap and was stored in a cold room ($4^{o}C$). Starch plates were stained for peroxidase activity as follows (Owen et al., 1958). o-Dianisidine (100 mg) was dissolved in absolute ethanol (70 ml). The resultant solution (7 ml) was mixed with acetate buffer (100 ml, 1 M, pH 4.7). Hydrogen peroxide (2 ml, 30%) was added to the mixture just before use. The sliced starch-gel was incubated (room temperature) in the mixture for 15 minutes, and was then washed three times with distilled water. The buffers used for electrophoresis were as follows:

Borate buffer (McKinley and Read, 1961)

Buffer A: A solution was prepared which was 0.3 molar in respect to boric acid and 0.6 molar in respect to sodium hydroxide. The reaction of the solution was pH 8.6. The buffer was used for the bridge compartments.

Buffer B: Boric acid (31 g) was dissolved in approximately 175 ml of sodium hydroxide solution (1N) and the volume of the resultant solution was made to 1L. The reaction of the final solution was pH 8.5.

Buffer C: Buffer B (23 ml) was diluted to 200 ml with distilled water. The reaction of the resultant solution was pH 8.6. The buffer was used in the preparation of the starch gel.

Tris and Borate Buffers (Poulik, 1957)

Tris (9.21 g) was dissolved in distilled water (950 ml) and citric acid (approximately 1.2 g) was added until the reaction of the solution was pH 8.6. The volume of the solution was adjusted to 1 L. This buffer was used in the preparation of the starch gel.

Boric acid (0.3 molar) was dissolved in distilled water (950 ml) and sodium hydroxide (ca. 0.1 molar) was added until the reaction of the solution was pH 8.6. The

volume of the solution was adjusted to 1 L. This buffer was used for the bridge compartments.

4. PREPARATION OF DOWEX 50 X-8 RESIN (200-400 MESH)

Resin (50 g) was placed in distilled water (500 ml) and the mixture was stirred for about 16 hours. It was then transferred to a sintered glass funnel and was washed with sodium hydroxide (2 L, 2 N) followed by distilled water until the wash water was neutral. The resin was then washed with hydrochloric acid (2 L, 2 N) followed by distilled water until the wash water was free of chloride ion. The resin that was to be used for chromatography was stored as a suspension in distilled water (1:2 W/V). The resin that was to be used in hydrolysis was washed twice with portions of hydrochloric acid (500 ml, 0.075 N) and was stored as a suspension in hydrochloric acid (0.075 N, 1:2 W/V).

5. HYDROLYSIS OF SAMPLE FOR THE DETERMINATIONS OF HEXOSAMINE AND HEXOSE

The sample (10 mg) was placed in a Pyrex test tube (12 x 100 mm) along with 5 ml of the resin suspension (II. 4.) and the tube was sealed. Hydrolysis (20 hours, 100°C) was carried out in a rotary oven (Blue M Electric

Company). The tube was cooled and the contents were transferred quantitatively to a glass column (1 x 30 cm) carrying a sintered glass filter at the bottom. The resin was washed with water and the wash water (12 ml) was collected in a 25 ml glass stoppered volumetric flask (volumetric flask No. 1). The resin was then washed with two 5 ml portions of hydrochloric acid (2 N) and the wash solutions which contained the hexosamines were collected in a second 25 ml volumetric flask (volumetric flask No. 2). The contents of the first volumetric flask were run through the resin column and the column was again washed with distilled water. The effluent was collected in the volumetric flask (volumetric flask No. 1) and the volume was adjusted to 25 ml. This solution was used for the determination of hexose. The column was then washed with two 5 ml portions of hydrochloric acid (2 N) and the effluent was collected in the second volumetric flask (volumetric flask No. 2) and the volume was adjusted to 25 ml with hydrochloric acid (2 N). This solution was used for the determination of hexosamine.

6. ESTIMATION OF HEXOSAMINE

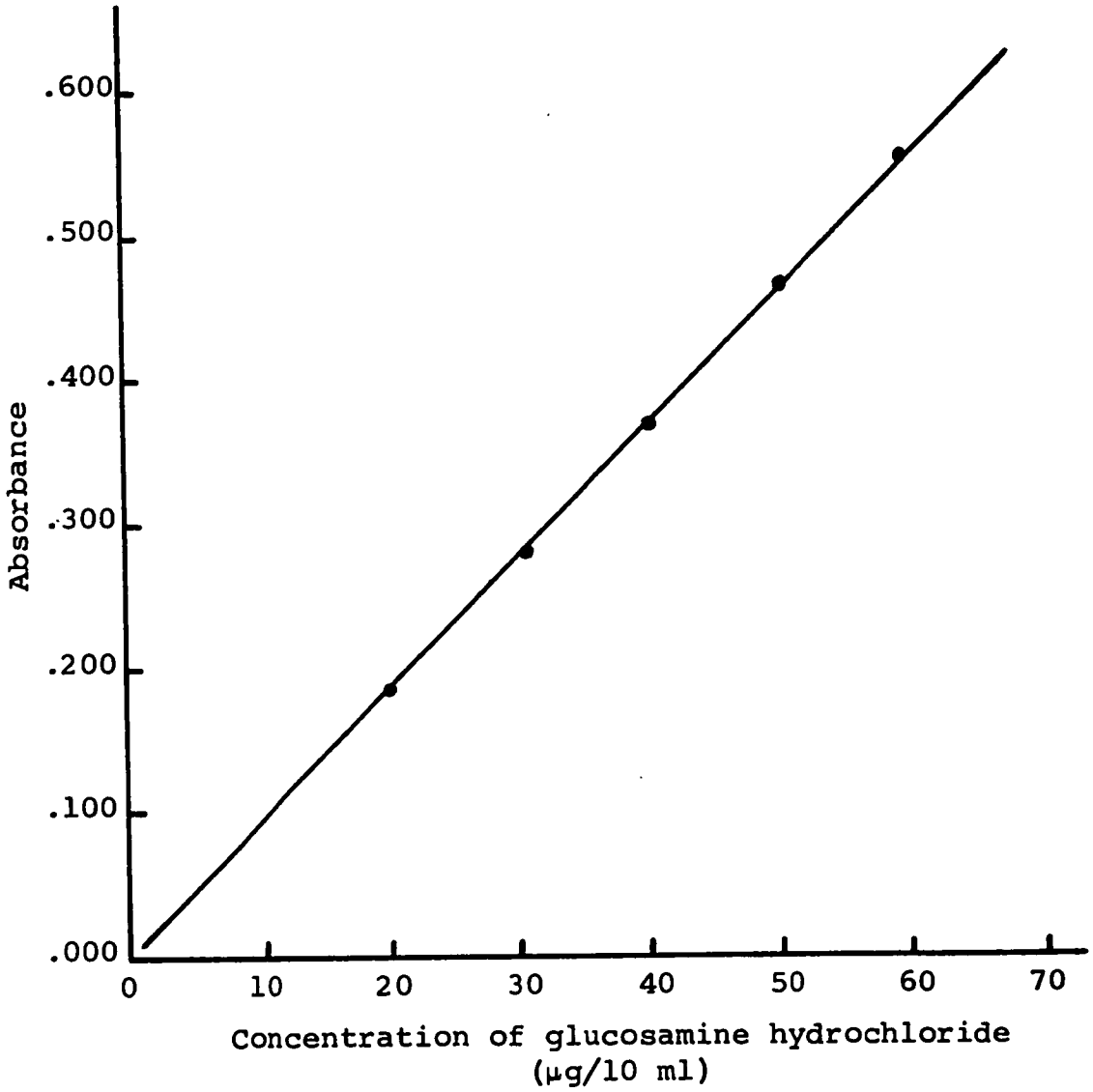
Hexosamine was determined by the method of Elson and Morgan (1933) as modified by Anastassiadis *et al*.

(1955). Aliquots of the acid eluate (II.5.) were placed in glass stoppered, volumetric flasks (10 ml) and were dried under vacuum in a desiccator containing sodium hydroxide flakes. The dried samples were redissolved in water (1 ml) and 2 ml of a 4% solution of acetylacetone in sodium carbonate (0.75 N) were added. The contents of the flasks were mixed thoroughly and were placed in a boiling water bath for one hour. The flasks were cooled in tap water for five to ten minutes. Absolute ethanol (5 ml) was added to each flask followed by 2 ml of Ehrlich's reagent. Ehrlich's reagent was prepared by dissolving p-dimethylaminobenzaldehyde (0.8 g) in absolute ethanol (30 ml) and the resultant solution was diluted with concentrated sulfuric acid (30 ml). The volume of each solution was adjusted to 10 ml with absolute ethanol, and its absorbance was measured at 530 mμ on a Beckman spectrophotometer. A calibration curve was prepared using glucosamine hydrochloride as a standard (Figure 2).

7. ESTIMATION OF TOTAL HEXOSE

Hexose was determined by the method of Fairbairn (1953). Aliquots of the water eluate (II.5.) were placed in glass stoppered, Pyrex tubes (16 x 150 mm). Anthrone reagent (10 ml) was added to each tube and the contents

Figure 2. Calibration Curve of Glucosamine Hydrochloride



were mixed thoroughly. The anthrone reagent was prepared by dissolving anthrone (1 g) in sulfuric acid (1 L). The acid was prepared as follows: concentrated sulfuric acid (700 ml) was added to water (200 ml) and the volume of the resultant solution was adjusted to 1 L by the addition of water. The tubes were heated in a boiling water bath (10 minutes) and were then cooled in tap water (5 minutes). The absorbances of the solutions were measured using a Beckman Spectrophotometer (620 mμ). A standard calibration curve was prepared using galactose as a standard (Figure 3).

8. ESTIMATION OF SIALIC ACID

The sample (10 mg) was placed in a Pyrex tube (12 x 100 mm) along with sulfuric acid solution (4 ml, 0.1 N), the tube was sealed and then was heated (80°C) for one hour in a rotary oven. The tube was cooled and opened, and the contents were transferred quantitatively to a 5 ml volumetric flask. The volume was adjusted to 5 ml, and the sialic acid content of the solution was determined by the method of Warren (1959). The calibration curve which was prepared using N-acetylneuraminic acid (Sigma Chemical Company) is shown in Figure 4.

9. THIN-LAYER CHROMATOGRAPHY (TLC)

The plates were prepared using a Quickfit thin layer

Figure 3. Calibration Curve of Galactose

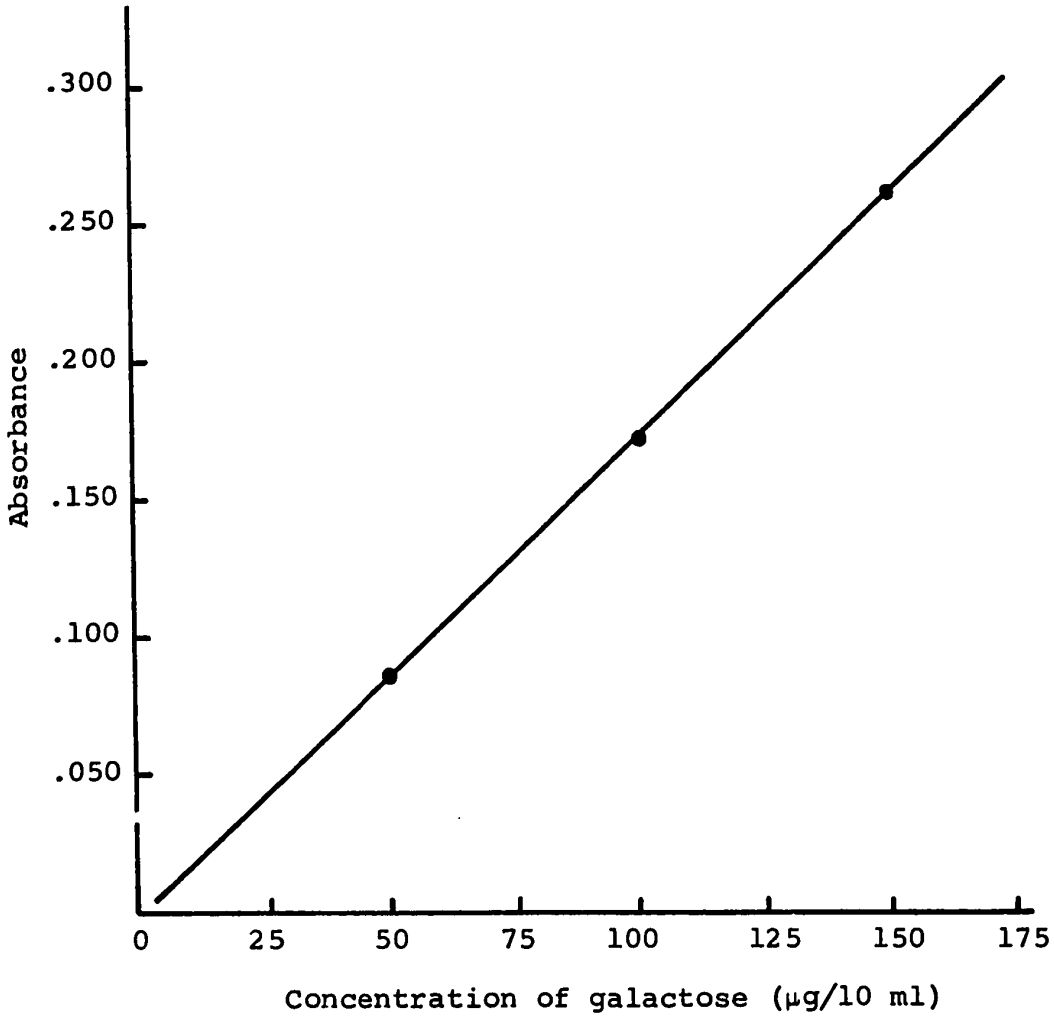
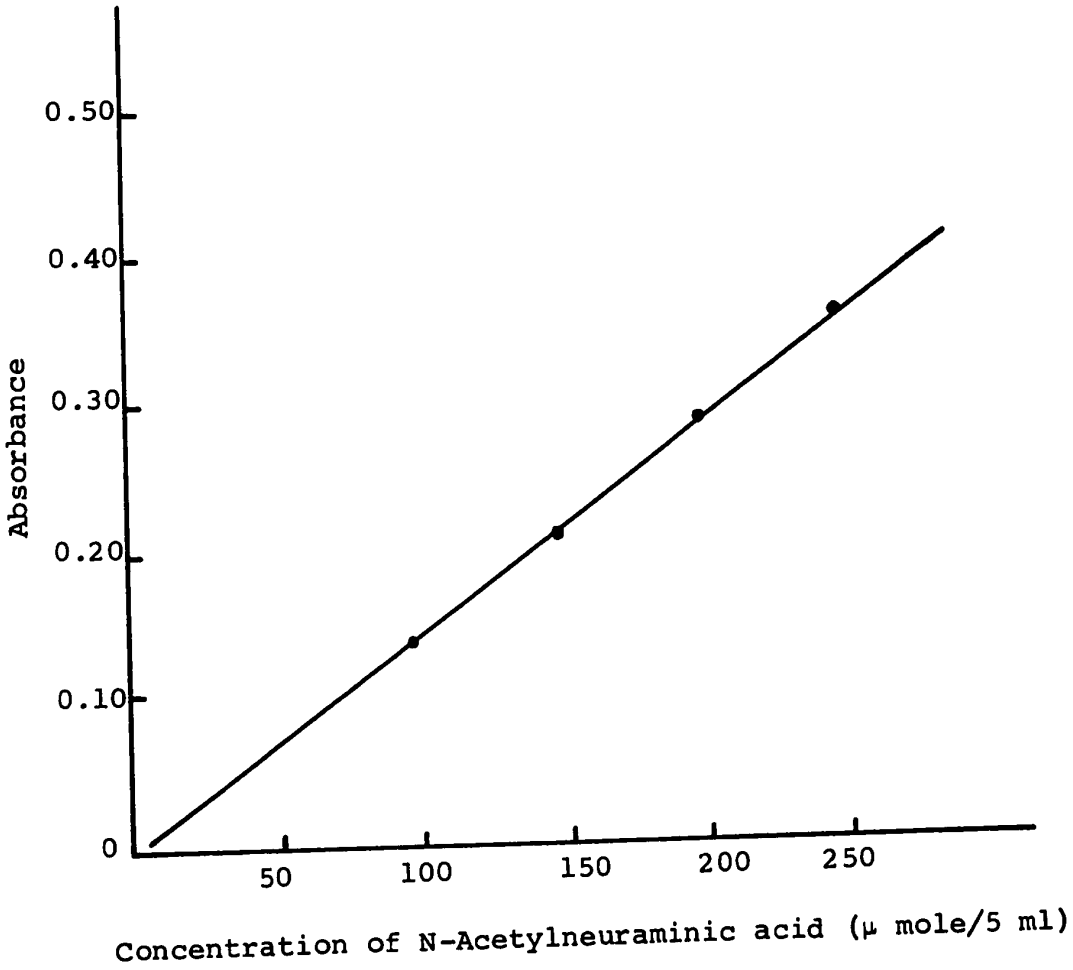


Figure 4. Calibration Curve of N-Acetylneuraminic Acid



spreader unit (Fisher Scientific Company). The basic technique for thin-layer chromatography is described by Stahl (1965).

10. ESTIMATION OF IRON

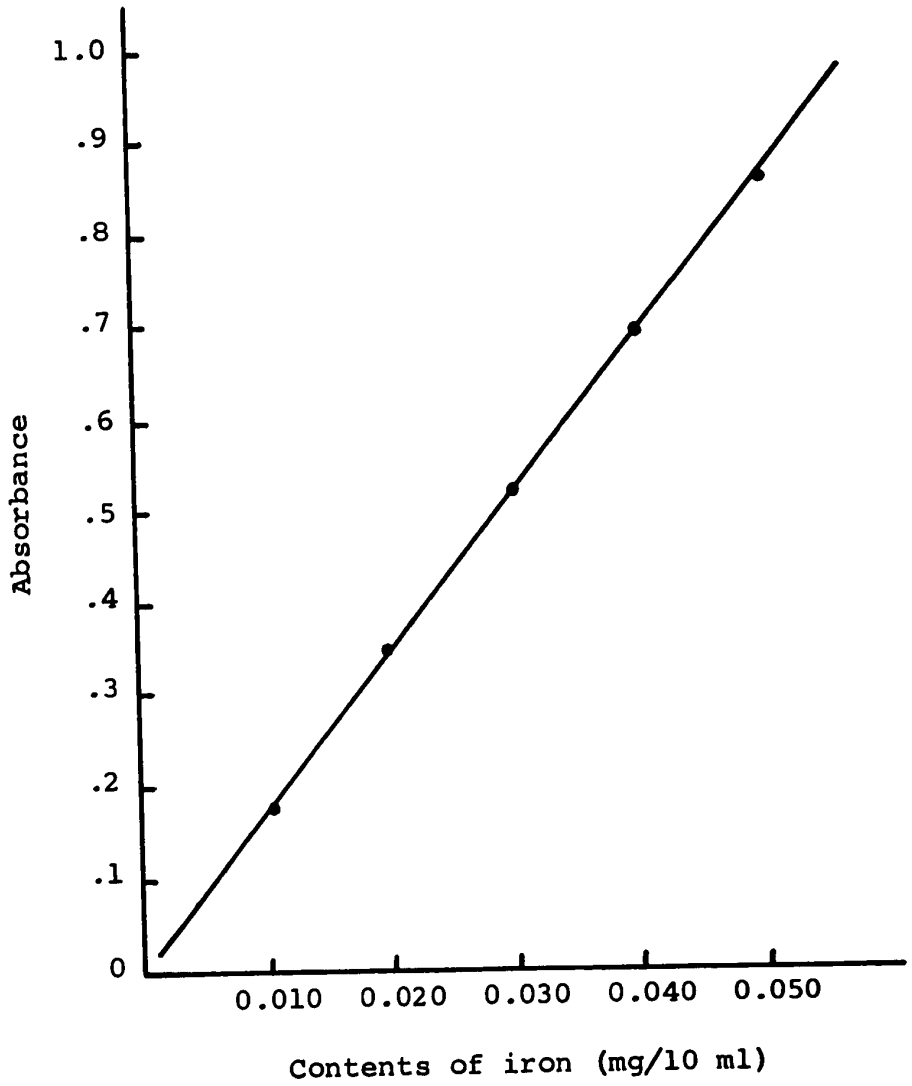
Iron was determined by the method of Delory (1943). The calibration curve which was prepared using a standard ferric ammonium sulfate solution is shown in Figure 5.

11. AMINO ACID ANALYSIS

Amino acids were determined by the method of Moore and Stein as described by Spackman et al. (1958).

The sample (5 mg) was placed in a Pyrex test tube (20 x 150 mm) along with constant boiling hydrochloric acid (5 ml, three times glass distilled). The mixture was frozen, the air was replaced by nitrogen, and the tube was sealed under vacuum. Hydrolysis was performed in a rotary oven at a temperature of 110°C for a period of time ranging from 24 to 96 hours. The tube was cooled to room temperature and the hydrolyzate was filtered through a small pre-washed Whatman No. 1 filter paper. The hydrolyzate was dried under vacuum (35°C) by means of a rotary evaporator. The dry residue was redissolved in a small volume of water and the solution was again dried in a rotary evaporator. The

Figure 5. Calibration Curve of Iron



procedure was repeated twice. The amino acid content of the sample was determined by means of a Beckman Spinco Model 120 C amino acid analyzer.

Tryptophan was determined spectrophotometrically by the method of Bencze and Schmid (1957).

12. IDENTIFICATION OF CARBOXYL-TERMINAL RESIDUES

The sample (30 mg) was dissolved in distilled water (30 ml) and the reaction of the solution was adjusted to pH 8.1 using sodium hydroxide (0.01 N). Carboxypeptidase C.T. (Nutritional Biochemical Corporation) (0.6 mg) was added to the solution and the mixture was incubated at 25°C. The reaction of the mixture was maintained at pH 8.0 $\pm$ 0.1 by the addition of sodium hydroxide solution (0.001 N). Portions (4 ml) of the solution were withdrawn after 5, 15, 30, 60, 120 and 180 minutes.

The reaction of each portion was adjusted immediately to pH 3 by the addition of hydrochloric acid (0.1 N) and the solutions were dialyzed against eight changes of hydrochloric acid (10 ml, 0.001 N). The diffusates from each digest were combined and evaporated under vacuum (35°C) in a rotary evaporator. The dry residue was redissolved in a small amount of distilled water and the solution was again dried. The procedure was repeated twice. The amino

acids in the diffusates were determined by means of Beckman Spinco Model 120 C amino acid analyzer.

13. PREPARATION OF DINITROPYRIDYL DERIVATIVES OF AMINO ACIDS IN PROTEINS (SIGNOR ET AL., 1965)

The sample (30 mg) was suspended in sodium bicarbonate solution (4 ml, 1%) and 2-chloro-3,5-dinitropyridine (5 ml, 1% in ethanol) was added to the solution. The mixture was stirred for two hours at room temperature, and was then acidified with concentrated hydrochloric acid followed by the addition of distilled water (5 ml). The mixture was extracted with several portions (5 ml) of ethyl acetate to remove ethanol, excess 2-chloro-3,5-dinitropyridine, and 2-hydroxy-3,5-dinitropyridine.

An equal volume of concentrated hydrochloric acid was added to the aqueous suspension of the insoluble dinitropyridyl-protein contained in a Pyrex tube (20 x 150 mm). The mixture was frozen, the air was replaced by nitrogen, and the tube was sealed under vacuum. The tube was heated in a rotary oven (110°C) for a period of time ranging from two to sixteen hours. An equal volume of distilled water was added to the resultant hydrolyzate which was then extracted with six portions (5 ml) of ethyl acetate. The combined ethyl acetate extracts were washed twice with

small portions of distilled water. The ethyl-acetate extracts were evaporated to dryness and the residues were dissolved in a small portion of acetone. The amino acid derivative in the acetone was analyzed by thin-layer chromatography.

14. PREPARATION OF DINITROPYRIDYL-AMINO ACID DERIVATIVE STANDARDS

Dinitropyridyl derivatives of 18 amino acids (alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, hydroxyproline, phenylalanine, serine, threonine, tryptophan, tyrosine, valine) were prepared by the following method.

The amino acid (10 mg) was suspended in sodium bicarbonate solution (2 ml, 1%), 2-chloro-3,5-dinitropyridine (4 ml, 1% in ethanol) was added and the mixture was shaken for two hours at room temperature. Distilled water (5 ml) was added to the reaction mixture and the mixture was extracted with several portions (5 ml) of ethyl acetate to remove ethanol, excess 2-chloro-3,5-dinitropyridine, and 2-hydroxy-3,5-dinitropyridine. The aqueous layer was acidified by the addition of a few drops of concentrated hydrochloric acid, and it was then extracted with several

portions (5 ml) of ethyl acetate. The ethyl acetate solutions of the dinitropyridyl amino acids were evaporated and the residues were stored in the dry state.

15. PREPARATION OF PHENYLTHIOHYDANTOIN DERIVATIVES OF AMINO ACIDS IN PROTEINS

The method that was employed was essentially the one described by Fraenkel-Conrat (1954).

The protein sample was dissolved in water (30 mg per ml) and the resultant solution was applied evenly to six strips (1 x 3 cm) of Whatman No. 1 filter paper by means of Pasteur pipette. Phenylisothiocyanate solution (1 ml, 20% in peroxide free dioxane) was applied to the paper strips in a similar manner. The strips were allowed to stand for four hours (40°C) in an atmosphere which was saturated with pyridine, dioxane, and water. The strips were then placed in a glass stoppered test tube along with benzene and the tube was gently shaken for three hours. The benzene in the test tube was replaced with fresh benzene every forty-five minutes. The paper strips were then washed for 24 hours using a mixture of ethanol and ether (1:1 V/V). The ethanol-ether mixture was replaced with a fresh one every hour.

The strips were removed from the tube and were

placed in a desiccator containing two beakers, one filled with hydrochloric acid (6 N) and another with glacial acetic acid. The desiccator was attached to a vacuum pump and was evacuated to a pressure of approximately 20 mm Hg. The strips were left in the desiccator (25°C) for 16 hours and they were then removed and were dried in a stream of air for one hour. The strips were placed in a glass stoppered test tube along with a mixture of ethanol and ether (1:1 V/V) and the tube was shaken gently for four hours. The ethanol-ether mixture in the test tube was replaced by fresh one every half hour during the extraction process. The extracts were combined and were then evaporated to dryness in a rotary evaporator (35°C). The residues were analyzed for phenylthiohydantoin amino acid derivatives by thin-layer chromatography.

16. PREPARATION OF GLOBIN (APO-MYOGLOBIN)

Globin was prepared from myoglobin by the method described by Lewis (1954). Myoglobin (15 mg) was dissolved in hydrochloric acid (1 ml, 0.1 N). The globin, which was split from the heme moiety by the acid treatment was precipitated by the addition of acetone (-20°C, 20 volume acetone per 1 volume of globin solution). The mixture was then centrifuged (0°C. 16,000 x g) for thirty minutes, and

the supernatant was discarded. The globin precipitate was dissolved in a small volume of distilled water and the reaction of the resultant solution was adjusted to pH 7, using sodium hydroxide solution (0.1 N). The globin solution was stored in the frozen state (-20°C).

III. EXPERIMENTAL

A. PRELIMINARY EXPERIMENT

1. PREPARATION OF SARCOPLASMIC EXTRACT

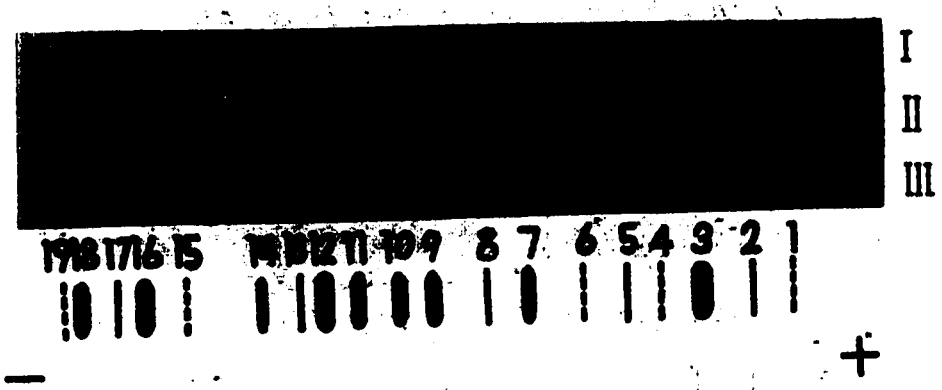
Ham muscle (Canada Packers, Montreal) was ground in distilled water (4°C. 1:1 W/V) by means of a Waring blender. The mixture was allowed to stand in a cold room (4°C) for one hour before it was centrifuged (30 minutes, 4°C, 16,000 x g). The supernatant was decanted and was filtered through Whatman No. 1 filter paper to remove fat droplets. The residue was ground again in the blender, and the mixture was centrifuged. The supernatant was filtered and the two filtrates were combined. The sarcoplasmic extract was freeze dried and was stored over sodium hydroxide flakes in a desiccator (4°C).

Starch-gel electrophoretic analysis of the sarcoplasmic extract showed that it contained at least 19 protein components (Figure 6). Buffer C (II. 3.) was used to prepare the starch gel.

2. PREPARATIVE STARCH-GEL ELECTROPHORESIS

An attempt was made to use preparative electrophoresis for the isolation of protein components from the sarcoplasmic

Figure 6. Electropherogram of Sarcoplasmic Extract in Starch-Gel (Borate Buffer pH 8.6)

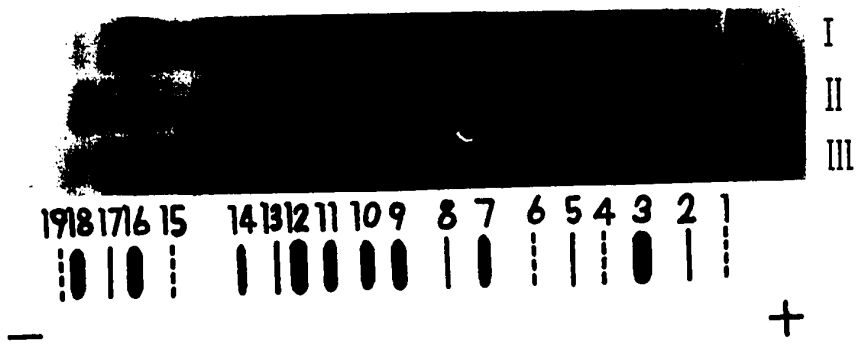


I sarcoplasmic extract from Vastus lateralis

II sarcoplasmic extract from Rectus femoris

III sarcoplasmic extract from Gracilis

Figure 6. Electropherogram of Sarcoplasmic Extract in Starch-Gel (Borate Buffer pH 8.6)



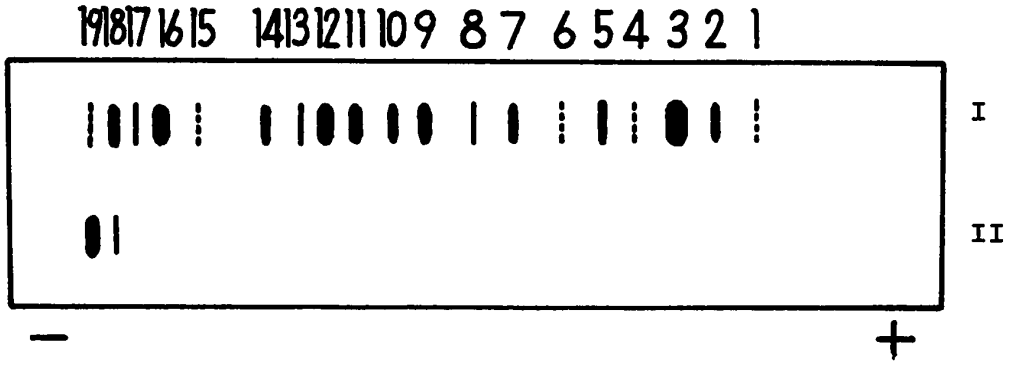
I sarcoplasmic extract from Vastus lateralis

II sarcoplasmic extract from Rectus femoris

III sarcoplasmic extract from Gracilis

extract. The same procedure was employed as is described in section II.3., except that the gel thickness was 1.8 cm instead of 0.6 cm. Sarcoplasmic extract (200 mg in 4 ml buffer C) was applied to the starch gel and after electrophoresis a small portion of the gel was cut out along the direction of electrophoresis. The starch gel, which was cut out as control, was sliced, stained, and washed. The section of the gel which corresponded to band 19 was cut out of the unstained portion of the gel. It was frozen, thawed, and the protein was eluted by squeezing out the buffer in the gel. It will be noted from Figure 6 that band 19 moved toward the cathode at pH 8.6, and hence was basic in nature. The isolated protein was subjected to electrophoresis using a 0.6 cm starch-gel plate and the electropherogram showed the presence of band 18 and band 19 (Figure 7). The total quantity of isolated protein was subjected to starch-gel electrophoresis (1.8 cm plate) and band 19 was cut out and the protein was isolated as described previously. Electrophoretic analysis of the isolated protein again showed the presence of band 18 and band 19. The protein of band 19 was subjected to preparative electrophoresis several times but it failed to separate band 19 from band 18.

Figure 7. Electropherogram of the Proteins (Band 19) Isolated by Preparative Starch-gel Electrophoresis (Schematic drawing of the electropherogram)



I Sarcoplasmic extract (see Figure 6)

II Fraction 19

During the course of this work Scopes (1966) reported the isolation of a basic protein from sarcoplasmic extract of pork muscle by a combination of acetone precipitation and column chromatography. Scopes (1966) showed that the basic protein component gave two bands when subjected to starch-gel electrophoresis. A sample of basic protein was prepared by the method of Scopes (1966) and its electrophoretic behaviour was compared to that of fraction 19 (p. 42) which was prepared by preparative electrophoresis. The experiment showed that Scopes' basic protein gave an electropherogram which was identical to that of fraction 19.

Scopes (1966) attempted unsuccessfully to separate the major component from the minor component by the use of an ion exchange column and other techniques. He noted that, on standing, the major component appeared to be converted to the minor component and on prolonged storage (0°C to 20°C) several minor components were present in the electropherogram. Ultracentrifugation of a preparation that had been stored for some time and which was shown by electrophoresis to contain five components, gave symmetrical sedimentation patterns. He concluded that the minor components are the slightly modified forms of the major component.

3. ISOLATION OF A PROTEIN COMPONENT BY SOLVENT PRECIPITATION AND COLUMN CHROMATOGRAPHY

A. PRELIMINARY EXPERIMENT

Sarcoplasmic extract was fractionated according to Scopes' (1966) method. Examination of the various fractions by starch-gel electrophoresis showed that the major component of one of the fractions had an electrophoretic mobility that was identical to band 5 (Figure 6). Band 5 of the sarcoplasmic extract (Figure 6) was brown in color before it was stained with amido black 10.B and was thought to be a myoglobin. However, the fraction obtained by Scopes' method had the same electrophoretic mobility as band 5 (Figure 6) and was colorless. The absorption spectrum of the fraction was measured between the range of 220 mμ to 700 mμ by the use of Spectronic 505 spectrophotometer. The fraction gave a single absorption maximum at 265 mμ.

B. ISOLATION OF FRACTION A

A procedure based on Scopes' method was devised for the fractionation of the sarcoplasmic extract.

Pork muscle (700 g) was placed in a Waring blender along with seven hundred milliliters of aqueous glycerol (3% v/v, 4°C) which contained 2 mM of EDTA. The muscle was

ground for one minute and the reaction of the mixture was adjusted to pH 6.5 by the addition of sodium hydroxide (1 N) and acetic acid (1 N). The amount of sodium hydroxide that was required for the pH adjustment depended upon the reaction of the meat slury. The quantity varied between 15 and 20 milliliters. The quantity of acetic acid that was required for the fine adjustment was between zero to two milliliters. The mixture was centrifuged for thirty minutes (0°C, 16,000 x g), and the supernatant was filtered through Whatman No. 1 filter paper. Cold acetone (-20°C, 43 ml per 100 ml extract) was added to the filtrate and the mixture was allowed to stand in the cold room (4°C) overnight. Additional acetone (-20°C, 35 ml per 100 ml original extract) was added slowly to the mixture and it was then centrifuged (0°C, 16,000 x g) for thirty minutes. Acetone (-20°C, 96 ml per 100 ml original extract) was added to the supernatant and the mixture was allowed to stand in the cold room (4°C) for one hour. The mixture was centrifuged (0°C, 16,000 x g) for thirty minutes and the supernatant was discarded. The precipitate was dissolved in a small amount of a sodium borate buffer (0.05 M in respect to borate, pH 6.8) and the protein solution was placed on a carboxymethyl-cellulose (Brown Co., Berlin,

N.H., U.S.A.) column (2.5 x 40 cm) which had been equilibrated with a sodium borate buffer (0.05 M in respect to borate, pH 6.8). The absorbed protein was desorbed with a sodium borate buffer (0.2 M in respect to borate, pH 8.5). The fractions which contained protein, were combined and the reaction of the solution was adjusted to pH 7.0 with saturated boric acid solution. Acetone (-20°C, 43 ml per 100 ml eluate) was added to the eluate and the mixture was allowed to stand in the cold room (4°C) overnight. Additional acetone (-20°C, 35 ml per 100 ml original eluate) was added slowly to the mixture and the mixture was centrifuged (0°C, 16,000 x g) for thirty minutes. Acetone (-20°C, 82 ml per 100 ml original eluate) was added to the supernatant and the mixture was allowed to stand in a cold room (4°C) for one hour. The mixture was centrifuged (0°C, 16,000 x g) for thirty minutes and the precipitate was discarded. The acetone in the supernatant was removed by the use of a rotary vacuum evaporator (10°C). The aqueous solution was dialyzed against distilled water and was then freeze dried. The freeze-dried protein was dissolved in a small amount of a borate buffer (Buffer C, II.3.), and the resultant solution was placed on a Sephadex-G75 column (1.5 x 40 cm) which had been equilibrated with the same

buffer. The protein was eluted (Buffer C,II.3.), at a flow rate of 20 ml per hour. The protein fractions that were obtained during the different stages of the fractionation procedure were examined by electrophoresis.

Figure 8 illustrates the fractionation procedure and Figure 9 shows the results of the electrophoretic analysis.

4. CHARACTERIZATION OF FRACTION A (FIGURE 8)

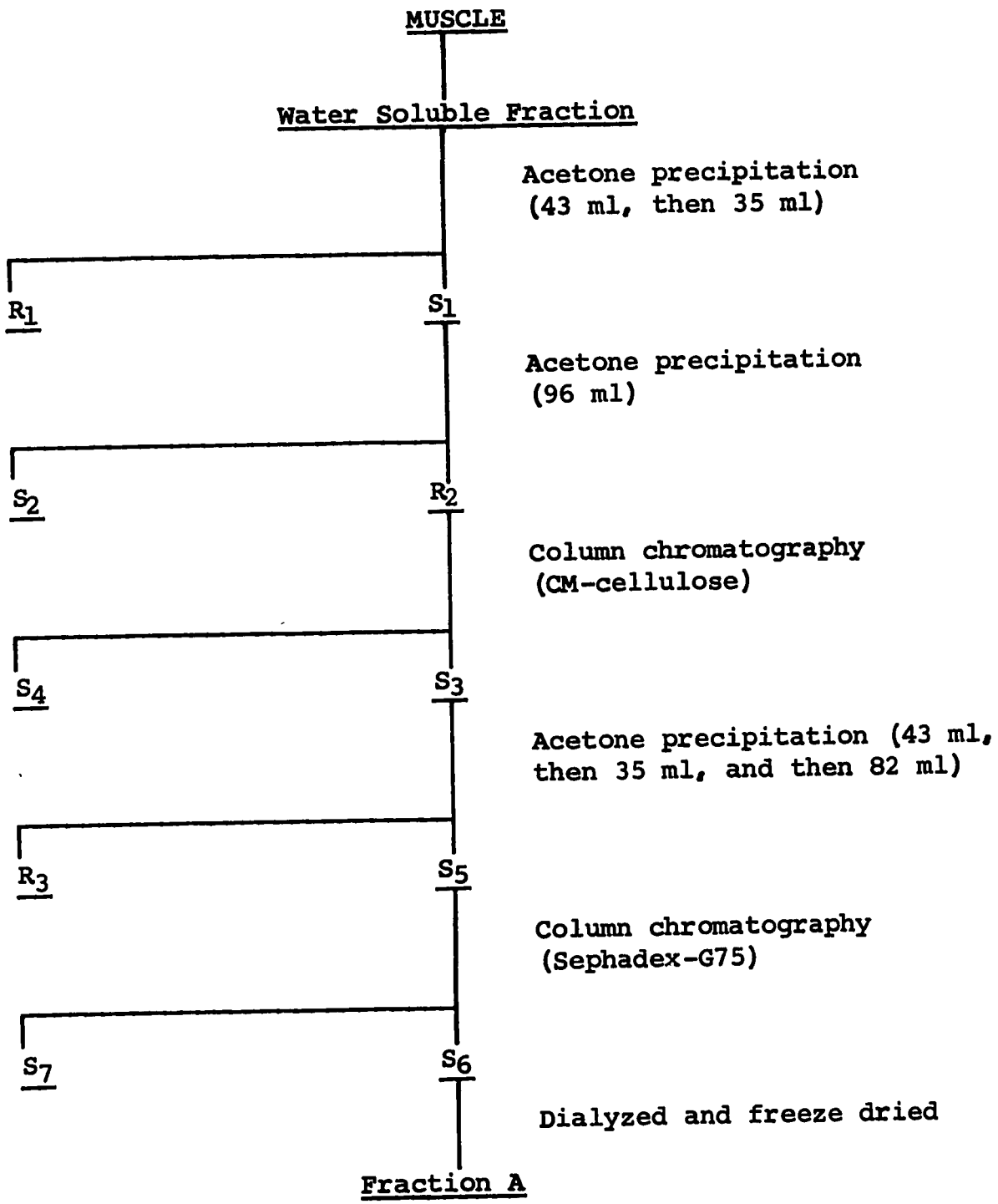
A. HOMOGENEITY

1) Starch-gel electrophoresis of Fraction A using the following two buffers showed that Fraction A was electrophoretically homogeneous.
   a) Buffer C (II.3.).
   b) Tris buffer (II.3.)
2) Fraction A was subjected to ultracentrifugation (Spinco Model E ultracentrifuge) in a sodium phosphate buffer (0.1 M in respect to phosphate, pH 7.0). The result showed that Fraction A was homogeneous. Figure 10 shows the result of the ultracentrifugation.

B. CHEMICAL ANALYSIS

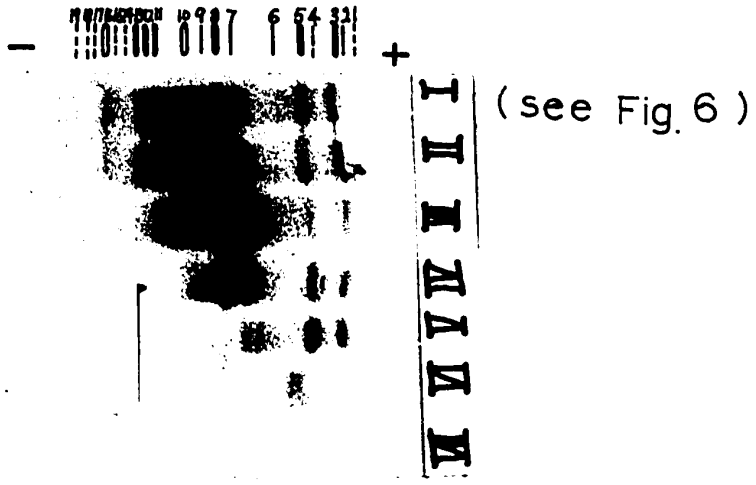
Table 1 shows the results of chemical analysis of Fraction A.

Figure 8. Schematic representation of fractionation procedure



S: solution R: residue

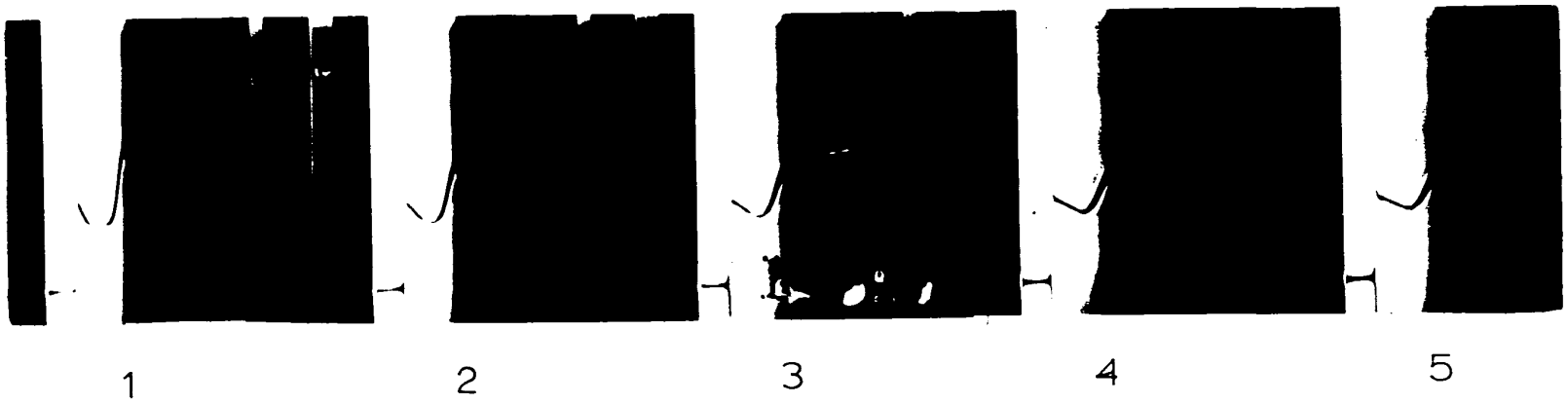
Figure 9. Electropherogram of the Fractions Obtained During the Preparation of Fraction A



I	Water soluble fraction*		
II	S_1*	V	S_5*
III	R_2*	VI	S_7*
IV	S_3*	VII	S_6*

*See Figure 8

Figure 10. Ultracentrifugal Pattern of Fraction A



1 pattern at 16 minutes after reaching speed
2 pattern at 24 minutes after reaching speed
3 pattern at 32 minutes after reaching speed
4 pattern at 40 minutes after reaching speed
5 pattern at 48 minutes after reaching speed

Table 1. Results of chemical analysis of Fraction A (moisture-free bases)

Total nitrogen (microkjeldahl)	13.36%
Total reducing sugar (as galactose)	13.53%
Hexosamine (as glucosamine hydrochloride)	1.26%
Sialic acid (as N-acetyl neuraminic acid)	0.089%
Iron	Nil

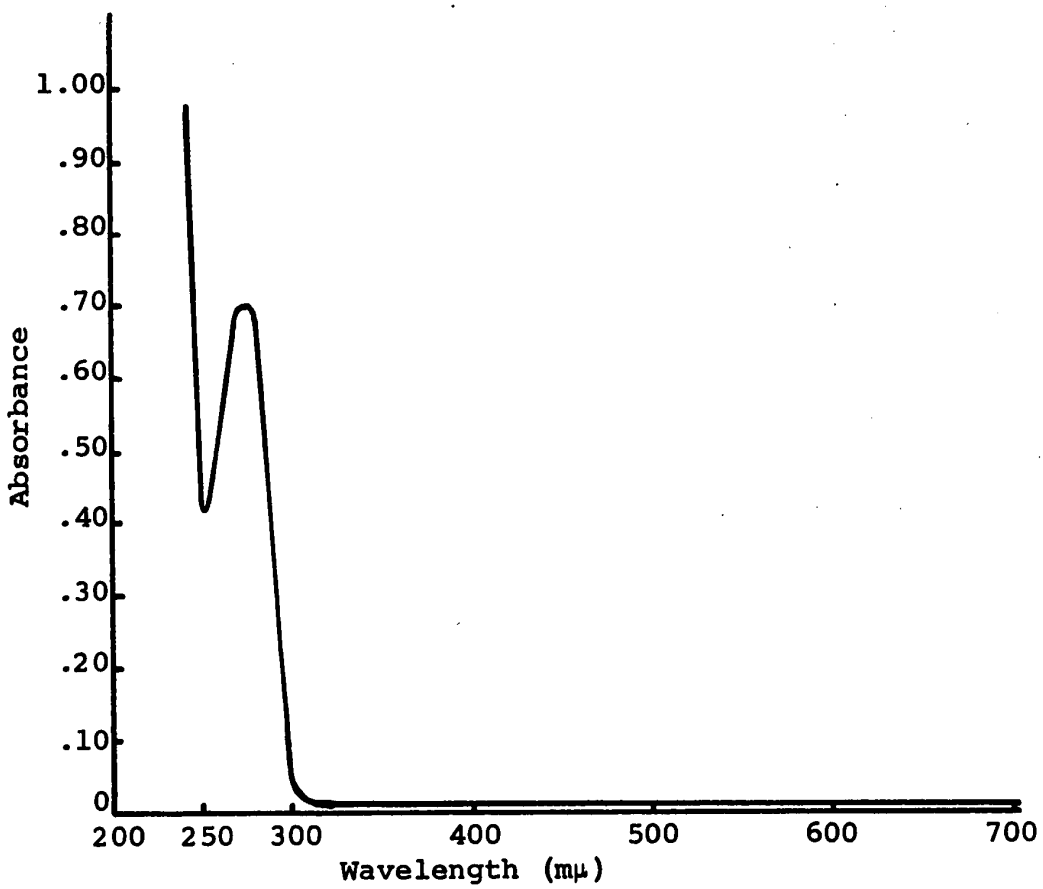
C. SPECTRAL ANALYSIS

The absorption maximum of Fraction A was measured by means of Spectronic Model 505 Spectrophotometer (220 mμ to 700 mμ). Figure 11 shows the result of the measurement. The result showed that Fraction A has a single absorption maximum at 265 mμ.

D. IDENTIFICATION OF AMINO ACID CONSTITUENT

The amino acids in a hydrolyzate (24 hours, 110°C) of Fraction A were identified by means of a Beckman Spinco Model 120 C amino acid analyzer. The following amino acids were identified: aspartic acid, threonine, serine, glutamic acid, glycine, proline, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine and arginine.

Figure 11. Absorption Spectrum of Fraction A (220 mμ to 700 mμ)



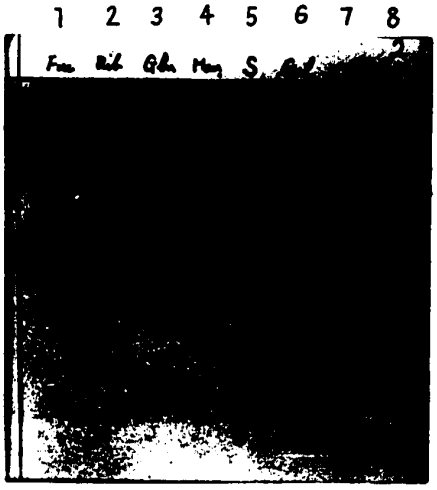
E. IDENTIFICATION OF SUGARS

Fraction A was hydrolyzed with Dowex 50 X-8 resin, and the hydrolyzate was fractionated into water eluate and acid eluate fractions. The water eluate was analyzed for sugar constituents.

a) By thin-layer chromatography

The water eluate (II.5.) was subjected to thin-layer chromatographic analysis using a Silica gel G plate prepared using boric acid (0.1N) (Pastuska, 1961). The spray reagent was prepared by mixing the following solutions: α-naphthol solution (10.5 ml, 15% solution in 95% ethanol W/V), concentrated sulfuric acid (65 ml), 95% ethanol (40.5 ml), water (4 ml). After the development of the chromatograms using suitable solvents, the plates were sprayed with the spray reagent and were then heated (100°C) for 15 minutes to develop the color. Figure 12 shows a typical chromatogram that was obtained using the solvent system methyl ethyl ketone-acetic acid-methanol (60:20:20 V/V). The result showed that the water eluate gave a major spot which corresponded to glucose. The faint streaking spot moved much more slowly than any of the standard sugars that were used and hence was not identified. Glucose was also identified using a solvent system made of

Figure 12. Thin-layer chromatogram of water eluate (Methyl ethyl ketone-Acetic acid-Methanol) 60 20 20



1. Fucose
2. Ribose
3. Glucose
4. Mannose
5. Water eluate
6. Galactose
7. Xylose
8. Fructose

ethyl acetate (65 ml) and a mixture (35 ml) of isopropanol and water (2:1 V/V).

b) By Paper Chromatography

The water eluate (II.5.) was subjected to paper chromatography using Whatman 3 MM chromatography paper and a solvent system composed of ethyl acetate-pyridine-water (12:5:4 V/V). The sugars were detected by use of the stain developed by Trevelyan et al. (1950). Figure 13 shows a typical paper chromatogram. The result showed that only glucose was present in the water eluate.

F. PREPARATION OF GLYCOGEN-APO-MYOGLOBIN COMPLEX

The results of the preceding experiments suggested that Fraction A might be a complex formed between apo-myoglobin and a glycogen fragment.

Myoglobin was prepared from pork muscle by ammonium sulfate fractionation (Ginger et al., 1954), and apo-myoglobin was prepared from the myoglobin by the method of Lewis (1954). The myoglobin and apo-myoglobin preparations along with Fraction A were subjected to electrophoretic analysis. The preparations had similar electrophoretic mobilities. The apo-myoglobin showed a single absorption maximum at 280 mμ when subjected to spectral analysis

Figure 13. Paper chromatogram of Water Eluate

1. Mannose
2. Glucose
3. Water eluate
4. Galactose
5. Fucose
6. Fructose

(220 mμ to 700 mμ; Spectronic 505 Spectrophotometer).

Glycogen was prepared from pork muscle as follows. Pork muscle (100 g) was ground in a trichloroacetic acid solution (100 ml, 10% TCA in water) by use of a Waring blender. The mixture was centrifuged (25°C, 10,000 X g, 10 minutes) and the precipitate was discarded. Ethanol (2 volumes, 95% ethanol) was added to the supernatant and the mixture was allowed to stand until a flocculent precipitate was formed and then it was centrifuged (25°C, 10,000 X g, 10 minutes). The supernatant was discarded and the glycogen precipitate was dissolved in a small amount of distilled water. The glycogen was reprecipitated by the addition of ethanol (2 volumes, 95% ethanol) and it was collected by centrifugation (25°C, 10,000 X g, 10 minutes). The reprecipitation procedure was then repeated.

The absorption spectra (220 mμ to 700 mμ) of the glycogen preparation was determined by means of a Spectronic 505 Spectrophotometer. The result showed that the glycogen preparation gave a single absorption maximum at 265 mμ.

When the apo-myoglobin preparation was mixed (ratio of glycogen/apo-myoglobin ranged from 0.1 to 10) with the

glycogen, the characteristic absorption maximum of the protein at 280 mμ shifted gradually toward that of the glycogen at 265 mμ with the increase of the glycogen/apo-myoglobin ratio. The shift of the absorption maximum of the mixtures was complete when the glycogen/apo-myoglobin ratio was larger than eight. The absorption maximum of Fraction A (265mμ) was unlikely the result of the overshadowing of the apo-myoglobin absorption maximum peak (280 mμ) by the glycogen absorption maximum peak (265 mμ) for the following reason. Two mixtures of glycogen and apo-myoglobin were prepared. One mixture had a glycogen/apo-myoglobin ratio of 0.1 and the other had a ratio of 0.2. The absorption maximum of each of the mixtures was at 280 mμ. It will be recalled that Fraction A had a carbohydrate content of 13.5% (Table 1) and this would give a carbohydrate/protein ratio of approximately 0.15.

Two samples of Fraction A were subjected to electrophoresis in polyacrylamide gel (Tris buffer, pH 8.6, II.3.). The gel was cut into two pieces and one was stained for protein (amido black 10B) while the other was stained for glucose (periodic acid-Schiff's stain, Keyser 1964). The results showed that the protein moiety migrated toward the anode during electrophoresis while the

sugar moiety remained at the starting line.

5. DISCUSSION

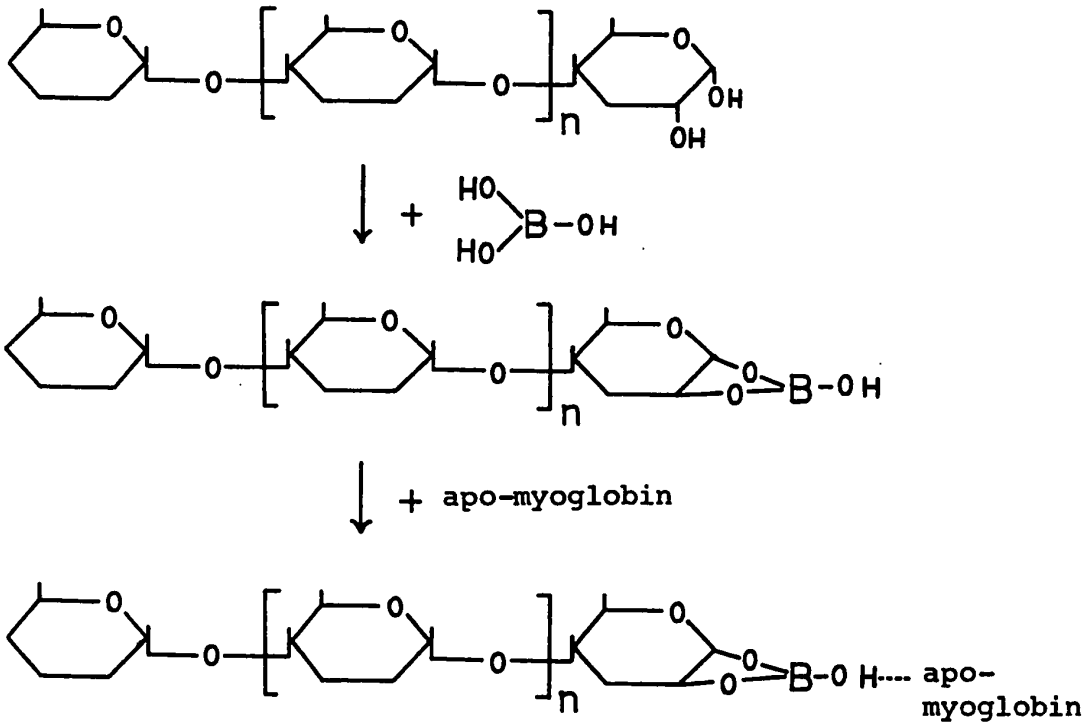
The fact that Fraction A contained about 13% nitrogen and 13% hexose would suggest that it might be a glycoprotein. However, the fraction contained only glucose as the sole hexose constituent and a small amount of sialic acid. Several preparations of Fraction A were made and each preparation had almost identical compositions.

The electrophoretic mobility of Fraction A was similar to that of myoglobin. However, Fraction A gave only one absorption maximum (265 mμ) between 220 mμ and 700 mμ, while myoglobin gives absorption maxima of approximately 410 mμ, 500 mμ and 630 mμ in the range of 350 mμ to 700 mμ (Smith and Gibson, 1959).

These experiments might suggest (a) that Fraction A comprises apo-myoglobin bound to a polyglucose unit in such a way that the absorption maximum of the complex is close to that of glycogen, and (b) that the complex which could have formed during the process of preparation of Fraction A, was dissociated under the condition of polyacrylamide gel electrophoresis.

There is the possibility that Fraction A is a complex of apo-myoglobin, a low molecular weight fragment of

glycogen and borate. The boron content of Fraction A determined by the carminic acid method (Demmitt, 1965) was 0.6 mg/g and it is well known that borate forms complexes with sugars (Boesken, 1949). Calculations based on the fact that Fraction A contains 83.5% protein (N X 6.25) and that apo-myoglobin has a molecular weight of approximately 17,000 show that the number of moles of boron per "molecular weight" of fraction A is 1.1. One might visualize that the protein-borate-carbohydrate complex was formed during the preparation of Fraction A in which borate was present in the buffer solution, as follows:



B. PORK MYOGLOBIN

Previous experiments showed that Fraction A had approximately the same electrophoretic mobility (starch gel, pH 8.6) as did myoglobin and apo-myoglobin. The amino acid composition of the protein moiety of Fraction A was similar to that of apo-myoglobin including the absence of cysteine. As little information is available on the chemical composition and physical properties of pure pork myoglobin it was decided to prepare crystalline pork myoglobin to gain precise information on its composition, constitution and physical properties.

1. PREPARATION OF MYOGLOBIN BY SALT PRECIPITATION

A sample of myoglobin was prepared by the method of Drabkin (1950) as modified by Ginger *et al*. (1954). Ham muscle was ground in distilled water (4°C, 1:1 W/V) by use of a Waring blender. The mixture was allowed to stand in a cold room (4°C) for one hour before centrifugation (4°C, 16,000 x g, 30 minutes). The supernatant was decanted and was filtered through Whatman No. 1 filter paper to remove fat droplets. The residue was ground again in distilled water, the mixture was centrifuged and the supernatant was filtered. The clear filtrates were combined (Fraction 1).

Saturated basic lead acetate solution (25 ml lead acetate solution per 100 ml extract) was added to the combined filtrate to precipitate the bulk of the proteins other than myoglobin. The mixture was centrifuged (4°C, 16,000 x g, 30 minutes), and potassium phosphate was added to the supernatant (Fraction 2) to adjust the phosphate ion concentration at 3 molar. This was accomplished by the addition of equimolar (in respect to phosphate) quantities of mono- and di-basic potassium phosphate. The reaction of the mixture was adjusted to pH 6.7 by the addition of potassium hydroxide solution (7 N). The precipitate was removed by centrifugation (4°C, 16,000 x g, 30 minutes), and the myoglobin solution (Fraction 3) was dialyzed against distilled water (4°C) to remove phosphate. Fraction 3 was dialyzed for 16 hours to give Fraction 4, and Fraction 4 was dialyzed for further 24 hours to give Fraction 5. A sample of myoglobin solution (Fraction 5) was treated with potassium ferricyanide to convert any oxymyoglobin that may have been present, into metmyoglobin. The following procedure was used: a few grains of potassium ferricyanide was added to a small portion (5 ml) of Fraction 5, and the mixture was allowed to stand (4°C) for one hour. It was then dialyzed against distilled water to remove potassium

ferricyanide (Fraction 6). Cyanmetmyoglobin was prepared by the addition of potassium cyanide (0.01% W/V) to a small portion (5 ml) of Fraction 5. The cyanmetmyoglobin solution was labelled Fraction 7. The absorption spectra (220 mμ to 700 mμ) of the various products (F1, F2, F3, F4, F5, F6, F7) were measured by means of a Spectronic 505 Spectrophotometer. Table 2 shows the results of the measurements and the absorption maxima (350 mμ to 700 mμ) of whale myoglobin reported by Smith and Gibson (1959).

Myoglobin in the muscle may be in several forms (e.g. oxy-, met-myoglobin) under the condition of the experiment.

The absorption maxima of Fraction 1 (630, 583, 542, 413, 280 mμ) as compared to that of whale oxymyoglobin (582, 545, 418 mμ) suggests that pork myoglobin is in the oxymyoglobin form in the muscle. The absorption maxima of Fractions 2, 3 and 4 remained unchanged, and this suggests that pork myoglobin stayed in the oxy form. The appearance of an absorption maximum at 500 mμ instead of 542 mμ and the shift in the absorption maximum at 413 mμ to 408 mμ in Fraction 5 suggest that the oxymyoglobin in this solution has been converted into metmyoglobin during the removal of phosphate by dialysis. Smith and Gibson (1959) (Table 2)

Table 2. Absorption maxima of different myoglobin preparations

Sample	Absorption maxima (mμ)
Oxymyoglobin	582, 545, 418 (1) 580, 540, 417, 282 (2)
Metmyoglobin	630, 500, 410 (1)
Metmyoglobin cyanide	540, 422 (1) 545, 424, 279 (2)
Sarcoplasmic extract (F1) (immediate after extraction)	630, 583, 542, 413, 280
Sarcoplasmic extract (F2) (after precipitation with lead acetate)	630, 583, 542, 413, 280
Myoglobin solution (F3) (after precipitation with 3 M phosphate)	630, 583, 542, 413, 280
Myoglobin solution (F4) (F3 dialyzed against H_2O for 16 hours)	630, 583, 542, 413, 280
Myoglobin solution (F5) (F3 dialyzed against H_2O for 40 hours)	640, 580, 500, 408, 280
Myoglobin solution (F6) (F5 treated with $K_3Fe(CN)_6$)	640, 580, 500, 408, 280
Myoglobin cyanide (F7) (added 0.01% KCN to F5)	540, 420, 280

(1) Data obtained from Smith and Gibson (1959)

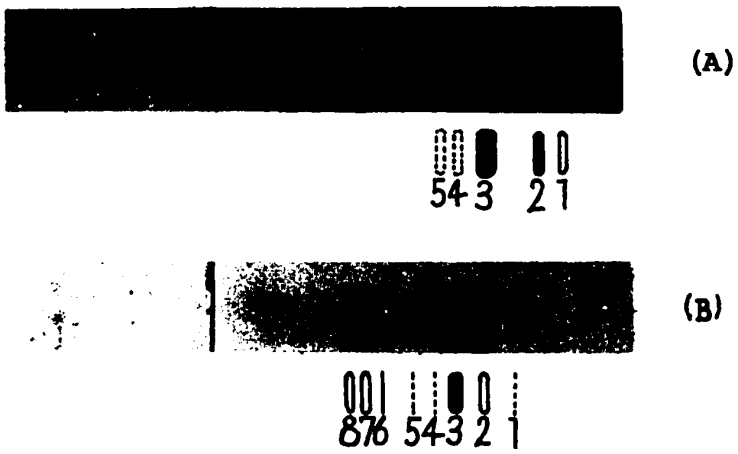
(2) Data obtained from Perkoff et al. (1962)

observed a shift in the absorption maxima at 545 and 418 mμ to 500 and 410 mμ respectively when oxymyoglobin (whale) was converted to metmyoglobin.

The myoglobin solution (Fraction 5) was freeze dried and the crude myoglobin preparation (Fraction 8) was stored over sodium hydroxide flakes in a desiccator ($4^{o}C$).

The myoglobin preparation (Fraction 8) was also examined by starch-gel electrophoresis using Tris buffer (II.3.) containing 0.1% potassium cyanide (II.3.). After electrophoresis the starch-gel was sliced and one half was stained with amido black 10 B (II.3.) for proteins and the other half was stained with o-dianisidine (II.3.) to detect substances having peroxidase activity (i.e. myoglobin). Figure 14 shows the results of the electrophoretic analysis. It will be noted that the bands in the gel which was stained with o-dianisidine did not match with those in the gel which was stained with amido black 10 B. The reason for this non-alignment is that the gel which was stained with amido black 10 B shrank when it was washed with methanol-water-acetic acid mixture whereas the other gel swelled slightly when it was washed with water. The bands 1, 2, 3, 4, 5, were more intensely stained with the

Figure 14. Electropherogram of a Myoglobin Preparation (Fraction 8) by Salt Precipitation



(A) Electropherogram stained with o-dianisidine

(B) Electropherogram stained with amido black 10B

Note: Gel B shrank due to the organic solvent wash. This accounts for the non-alignment of the bands in the two gels.

o-dianisidine than they were with amido black 10B. The electropherogram that was stained with o-dianisidine shows that pork myoglobin preparation comprised at least four minor heme protein components (bands 1, 2, 4, 5) and one major heme protein component (band 3). The electropherogram that was stained with amido black 10B shows that there were at least three non-heme proteins (bands 6, 7, 8) in the myoglobin prepared by salt precipitation. Lewis and Schweigert (1955) found by electrophoretic analysis that there were at least four proteins other than myoglobins which were precipitated along with myoglobins when the percent saturation of ammonium sulfate in the protein solution ranged from 85% to 100%.

2. CRYSTALLIZATION OF MYOGLOBIN

An attempt was made to prepare crystalline myoglobin from a myoglobin preparation (Fraction 8) obtained by salt precipitation.

The following methods were tried.

A. MODIFICATION OF THE METHOD OF LEWIS AND SCHWEIGERT (1955)

Solid ammonium sulfate (564.8 g/L) was added to the myoglobin solution (Fraction 8, 30 mg/1 ml) and the mixture was centrifuged (0°C, 16,000 x g, 30 minutes) to remove

insoluble material. Solid ammonium sulfate (70.6 g/L) was added to the clear supernatant and the mixture was again centrifuged (0°C, 16,000 x g, 30 minutes). The myoglobin which remained in the supernatant (Fraction 9) was precipitated by one of the following methods.

a) Saturated ammonium sulfate solution (4°C, pH 6.7) was added dropwise to the supernatant (Fraction 9) until the point of incipient cloudiness was noted. The mixture was allowed to stand in a cold room (4°C) overnight. The mixture was centrifuged and a brown amorphous precipitate was obtained. The precipitate was removed and additional saturated ammonium sulfate solution was added to the supernatant, the mixture was allowed to stand in a cold room and again an amorphous precipitate was obtained. The procedure was repeated until the supernatant solution was colorless. No crystalline myoglobin was obtained in this experiment.

b) Solid ammonium sulfate was added to the supernatant (Fraction 9) and the mixture was allowed to stand in a cold room (4°C) for about 16 hours. No crystalline myoglobin was noticed when the precipitate was examined by means of an ultraphotomicroscope (Karl Zeiss, Germany) (up to 1,000X). The process was repeated and this time the mixture was allowed to stand for one week but no

crystalline myoglobin was noticed.

c) The supernatant (Fraction 9) was dialyzed against a saturated ammonium sulfate solution (4°C, pH 6.7) which contained excess solid ammonium sulfate. The ammonium sulfate solution was agitated slowly during the dialysis (one week). Small portions of the brown precipitate which was obtained in the dialysis tube were taken at 24-hour intervals and were examined under the microscope. No crystals of myoglobin were observed.

B. THE METHOD OF LUGINBUHL (1960)

Solid ammonium sulfate (564.8 g/L) was added to a myoglobin solution (Fraction 8, 30 mg/ml) and the reaction of the mixture was adjusted to pH 8.0 by the addition of ammonium hydroxide (concentrated). The mixture was centrifuged (0°C, 16,000 x g, 30 minutes) to remove insoluble material. Ammonium sulfate was added to the supernatant until the solution was saturated in respect to ammonium sulfate and the reaction of the mixture was maintained at pH 8.0 by use of ammonium hydroxide (concentrated). The mixture was stirred gently overnight in a cold room (4°C) and was then centrifuged (0°C, 39,000 x g, 30 minutes) to remove insoluble material. A small amount of solid ammonium sulfate was added to the supernatant and the mixture

was stirred gently for four hours before centrifugation (0°C, 39,000 x g, 30 minutes). The reaction of the supernatant was adjusted to pH 6.7 by the addition of acetic acid (2% solution) and the resultant solution was allowed to stand at 4°C overnight. No myoglobin crystal was obtained by this method.

Atassi and Saplin (1966) adapted successfully the method of Luginbuhl (1960) for the preparation of crystalline human myoglobin to the preparation of crystalline finback myoglobin. The method of Luginbuhl (1960) is based on the fact that human myoglobin is soluble in saturated ammonium sulfate solution at pH 8.0, and myoglobin is subsequently crystallized out from the solution when the reaction of the solution is lowered to pH 6.7 (iso-electric point of myoglobin). Pork myoglobin, however, was completely precipitated from a saturated solution of ammonium sulfate even at pH 8.0. This would indicate that there might be species differences between pork myoglobin and the other myoglobins (e.g. human and finback whale myoglobins) which were crystallized according to Luginbuhl's method.

Several workers (Morgan, 1936; Roche et al., 1942; Lewis and Schweigert, 1955) have experienced similar difficulties in the crystallization of pork myoglobin. Morgan (1936) pointed out that there are species differences

between the various myoglobins which make some myoglobins more difficult to crystallize than others. Kendrew et al. (1954) reported that the myoglobins of the common domestic animals are difficult to crystallize. Rossi and Travia (1941) prepared crystalline pork myoglobin from cardiac muscle. The authors claimed that the crystals were large and belonged probably to the rhombic system. They used a method published previously (Rossi, 1940) which involved salt precipitation.

The failure to obtain crystalline myoglobin from Fraction 8 might have been the result of non-heme protein (bands 6, 7, 8, Figure 14) interference. An attempt was therefore made to remove these contaminants by column chromatography.

3. PURIFICATION OF MYOGLOBIN BY COLUMN CHROMATOGRAPHY

Theorell and de Duve (1947) first showed that crystalline myoglobin (human) was heterogeneous when it was subjected to electrophoresis (Tiselius). The heterogeneity of other crystalline myoglobins was reported by other workers (Schmid, 1949; Lewis, 1954).

Boardman and Adair (1956) separated crystalline horse myoglobin into two fractions by column chromatography

(Amberlite IR-50 resin). Later seal (Rumen, 1959), sperm whale (Edmundson and Hirs, 1961), human (Perkoff et al., 1962), finback whale (Atassi and Saplin, 1966), harbor seal and porpoise (Hapner et al., 1968) myoglobins were each separated column chromatographically into five or more fractions by the use of CM-cellulose, Amberlite IR-50 resin, DEAE-cellulose, CM-cellulose and CM-Sephadex, respectively. The author chose CM-cellulose for the column chromatography of pork myoglobin.

Carboxymethyl cellulose (100 g) was suspended in distilled water and the mixture was stirred overnight. The cellulose was transferred to a Büchner sintered glass funnel and was washed with sodium hydroxide (1 L, 0.2 N) then with hydrochloric acid (1 L, 0.2 N) and finally with distilled water until it was free of chloride ion. The washed cellulose was suspended in a phosphate buffer (pH 6.2, 10 mM in respect to phosphate and containing 0.1 g KCN per liter). The suspension was stirred and was allowed to stand (25°C) for twenty minutes. The fine particles which remained suspended in the supernatant were removed by decantation. The process was repeated until the supernatant was clear. A sufficient quantity of cellulose suspension was transferred to a column chromatographic

tube (3.5 x 70 cm) to form a cellulose column of approximately 60 cm in height. The column was equilibrated by washing it with the phosphate buffer solution (same as above) until the reaction of the effluent was the same as that of the buffer.

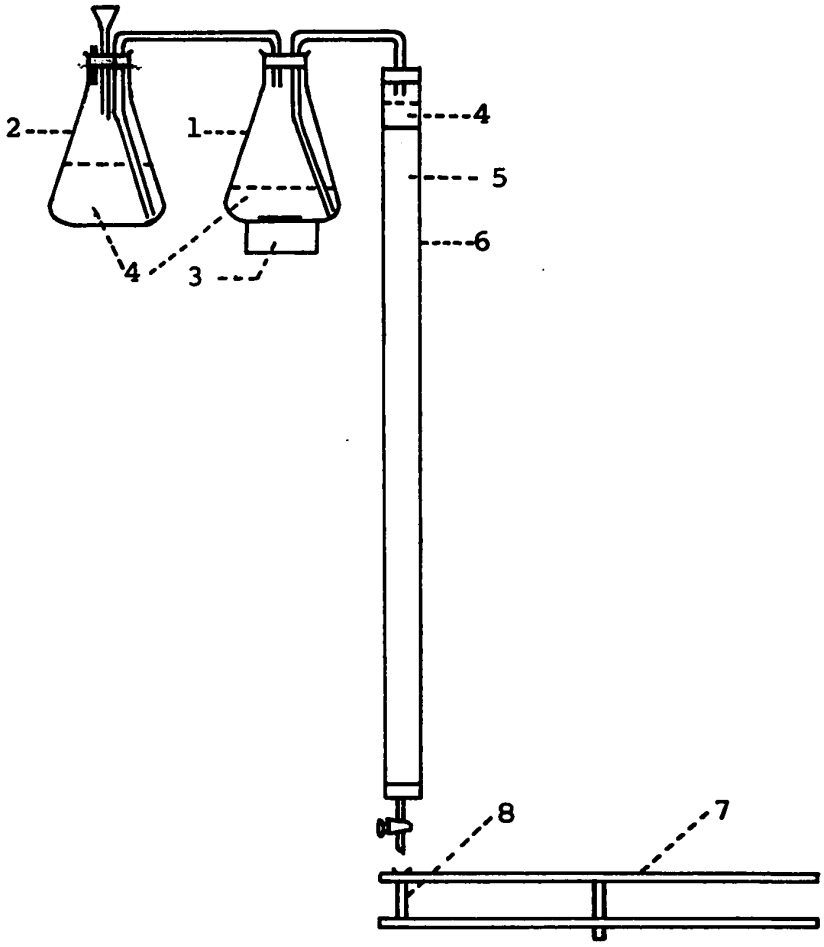
The cellulose column was mounted above a fraction collector (LKB 3,400 B Radi Rac Fraction Collector, LKB-Produkter A B, Stockholm) in a cold room (4°C). Figure 15 shows a diagram of the assembly.

Myoglobin (300 mg, Fraction 8) which was dissolved in a phosphate buffer (10 ml, pH 6.2) was placed on the column and the proteins were eluted from the column as follows. Phosphate buffer (20 ml, pH 6.2, 10 mM in respect to phosphate and containing 0.1 g KCN per liter) was placed in chamber 1 (Figure 15) and the same buffer (130 ml) was placed in chamber 2 (Figure 15). The buffer was passed through the column at a rate of 20 ml per hour. When chamber 2 was nearly empty, another buffer was placed in chamber 2. The buffers which were placed successively in chamber 2 were as follows:

120 ml phosphate buffer (10 mM, 0.1 g KCN/L)	pH 7.0
250 ml phosphate buffer (10 mM, 0.1 g KCN/L)	pH 7.2
280 ml phosphate buffer (10 mM, 0.1 g KCN/L)	pH 7.3
200 ml phosphate buffer (10 mM, 0.1 g KCN/L)	pH 7.7

The eluate was collected in 15 ml fractions and the

Figure 15. Assembly of Column Chromatography



1	Buffer chamber 1	5	CM-Cellulose
2	Buffer chamber 2	6	Glass column
3	Magnetic stirrer	7	Fraction collector
4	Buffer solution	8	Test tube

absorbance of each fraction at 280 mμ and 420 mμ was measured by means of a Beckman D.U. Spectrophotometer. The 15 ml fractions which belonged to the same peak were pooled, and the absorption spectra of the pooled solutions were measured (Spectronic 505 Spectrophotometer) over the range of 220 mμ to 700 mμ. The pooled solutions were also examined by starch-gel electrophoresis.

Figure 16 shows the result of the column chromatography of the myoglobin preparation. The myoglobin preparation was fractionated into at least five fractions (P1, P2, P3, P4, P5). Table 3 shows the results of spectral analysis of the pooled solutions. All of the solutions had similar absorption maxima between 220 mμ to 700 mμ. The slight shift of the absorption maxima of P1 and P2 toward a shorter wavelength (532 and 412 mμ instead of 540 and 420 mμ of P3, P4, P5) was most probably due to the lower pH of the solutions. The lower ratios of E_{420}/E_{280} of P1, P2, P4 and P5 suggested that these fractions were contaminated with non-heme proteins. This was confirmed by starch-gel-electrophoretic analysis (Figure 17).

The percentage of the total protein that was present in each of the fractions (P1, P2, P3, P4, P5) was estimated from the extinction coefficients (E_{280}) which

Figure 16. Column Chromatography of Myoglobin by CM-cellulose

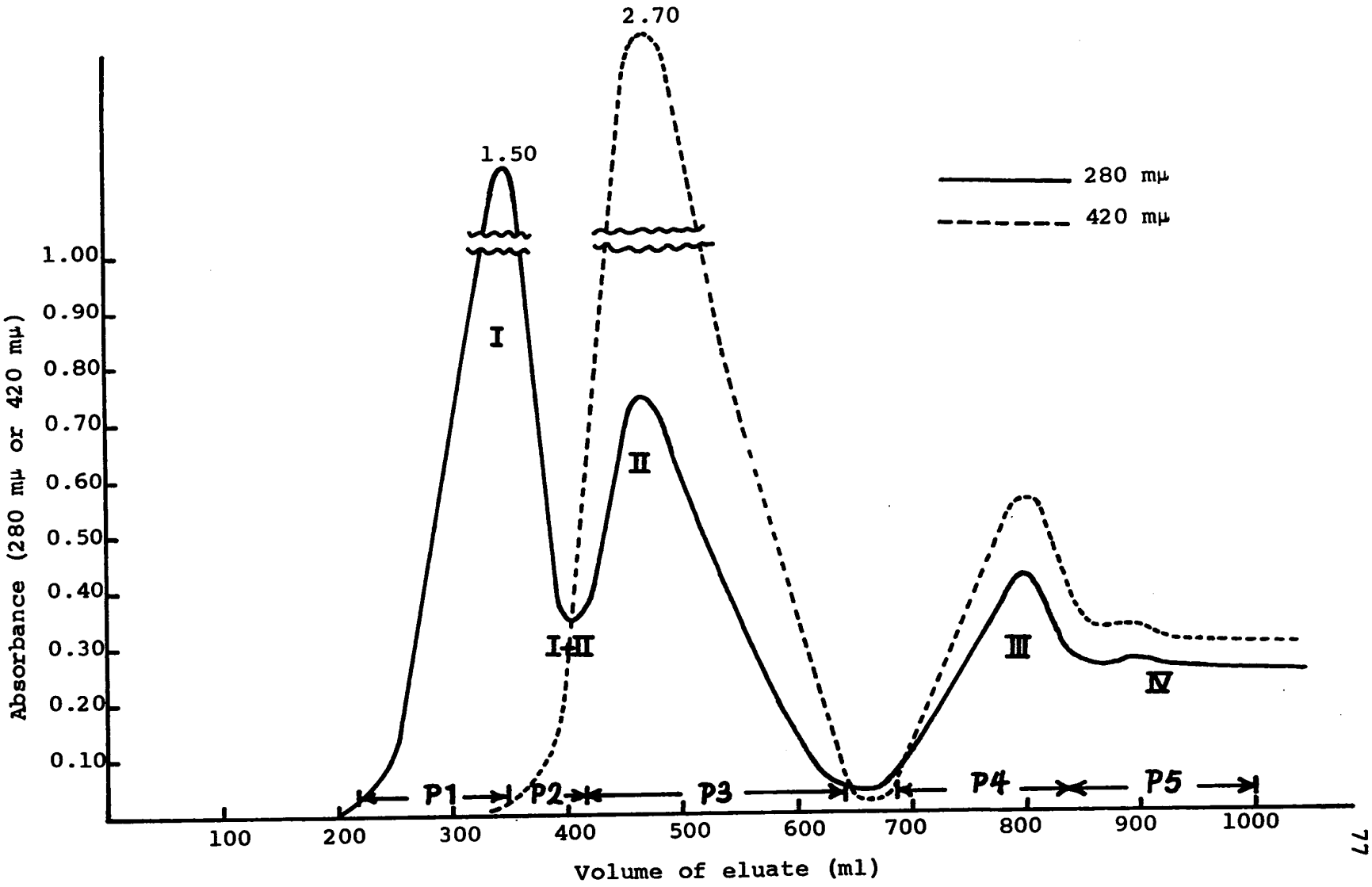


Table 3. Absorption Maxima of the Column Chromatographic Fractions

Fractions	Absorption Maxima (mμ)	E_{420}/E_{280}	E_{280} x Volume
P1	532 412 350 280	0.16 (1)	54.0
P2	532 412 350 280	1.08 (1)	19.0
P3	540 420 355 280	3.50	85.5
P4	540 420 355 280	1.15	29.3
P5	540 420 355 280	1.56	7.5

(1) E_{412}/E_{280} instead of E_{420}/E_{280}

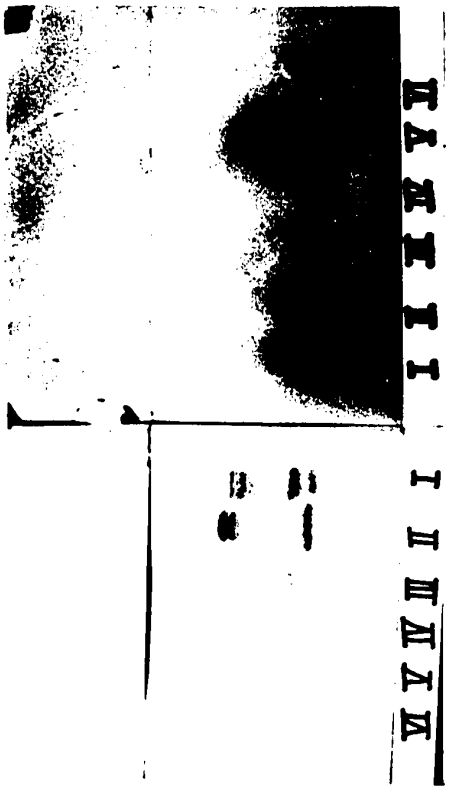
were calculated from the optical density (280 mμ) of the eluate fractions (Atassi and Saplin, 1966). The following values were obtained: P1, 27.5%; P2, 9.6%; P3, 43.3%; P4, 14.9%; P5, 3.8%. The recovery of proteins from the column, based on the extinction at 280 mμ, was about 96%.

Figure 17 shows the results of starch-gel-electrophoretic analysis of the fractions.

P1 comprised three heme proteins (bands 1, 2, 3) and three non-heme proteins (bands 6, 7, 8) with a predominance of non-heme proteins. This observation was supported by the low E_{412}/E_{280} ratio of the fraction (Table 3).

P2 was probably a mixture of P1 and P3. It contained all the electrophoretic components of P1 with the exception

Figure 17. Electropherogram of fractions P1 to P5



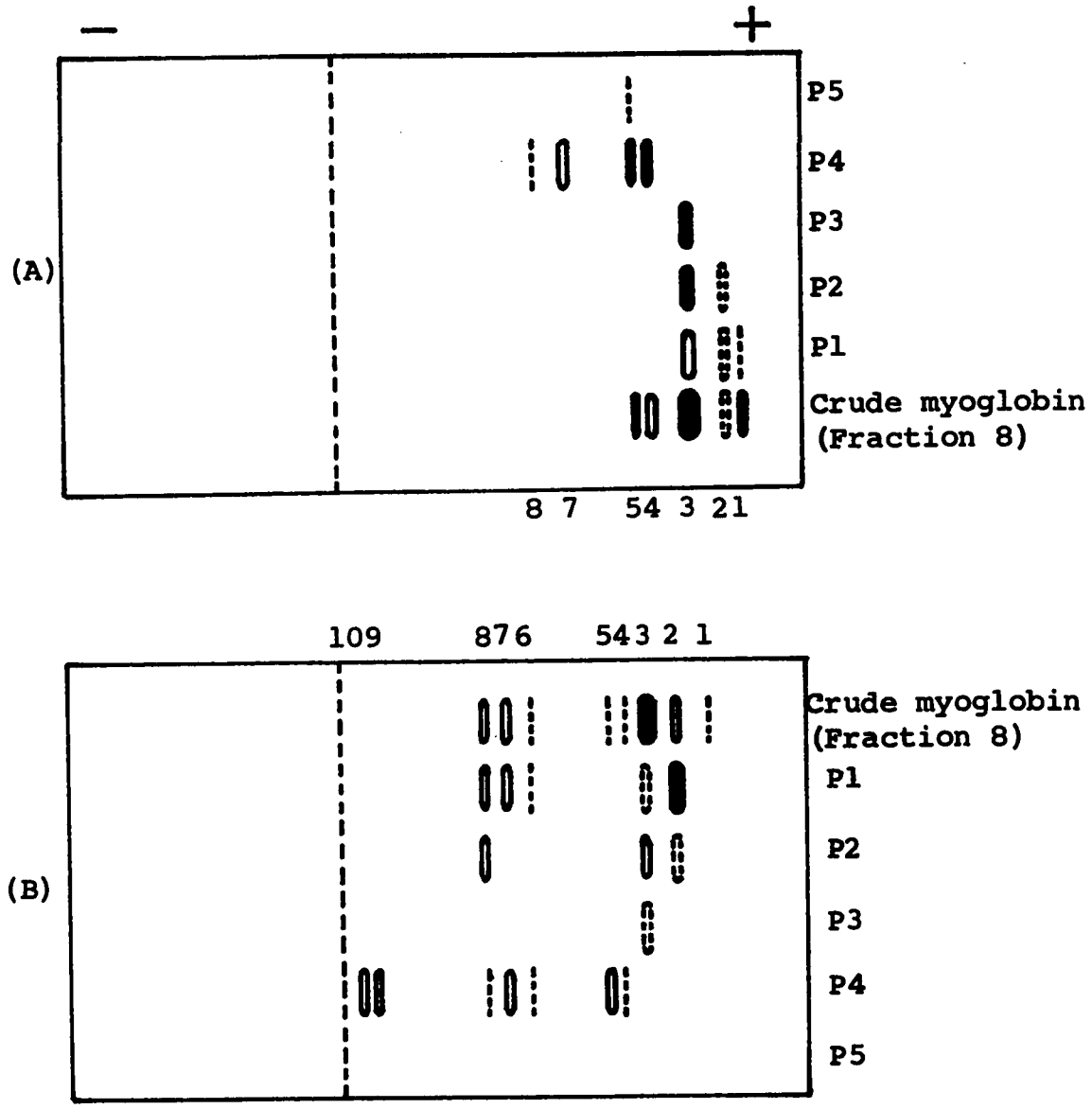
(A)

(B)

(A)	o-dianisidine stained	(B)	amido black 10B stained
I	crude myoglobin	IV	P3
II	P1	V	P4
III	P2	VI	P5

Note: Gel B shrank due to the organic solvent wash. This accounts for the non-alignment of the bands in the two gels.

Figure 17. Schematic Representation of the Electropherogram of fractions P1 to P5



(A) o-dianisidine stained (B) amido black 10B stained

Note: Gel B shrank due to the organic solvent wash. This accounts for the non-alignment of the bands in the two gels.

of bands 1 and 8 which might have been presented in such low concentration that they were not detected.

P3 gave a single electrophoretic component on the electropherogram that was stained with o-dianisidine and on the one that was stained with amido black 10B. The band in each of the electropherograms of P3 corresponded to the major band (band 3) of the myoglobin preparation (F.8). The E_{420}/E_{280} ratio of P3 was 3.5.

P4 comprised four slow moving heme proteins (bands 4, 5, 7, 8) and three slow moving non-heme proteins (bands 6, 9, 10). It appeared that the heme proteins were present in higher concentration than were the non-heme proteins.

Electrophoretic analysis of P5 showed that it contained one electrophoretic component (band 5, Figure 17A). However, the low E_{420}/E_{280} ratio (Table 3) of this fraction suggested that P5 contained some non-heme protein.

It will be recalled from the results reported in Figure 14 that o-dianisidine produced a more intense color with a given heme protein than did amido black 10B. This is also evident if one compares the color intensity of band 3 (P1) of gel A and gel B in Figure 17. As band 2 (P1) in gel B is more intense than is band 2 (P1) in gel A, one might conclude that the protein in this band

comprises non-heme protein as well as heme protein.

The non-heme proteins (bands 7, 8) were found in the amido black 10B stained electropherogram of crude myoglobin (III.B.1.) and P1 fraction which was obtained by the fractionation of crude myoglobin. However, heme protein was not found in the corresponding position on the electropherogram which was stained with o-dianisidine. On the other hand, two heme proteins which gave a similar electrophoretic mobility to those of bands 7 and 8, were detected on the electropherogram (o-dianisidine stained) which was prepared from the P4 fraction. These results indicate that the crude myoglobin preparation contains two heme proteins and two non-heme proteins which have electrophoretic mobilities that are similar to those of bands 7 and 8, respectively. Thus the positions which correspond to bands 7 and 8 could be occupied by a heme protein or a non-heme protein or a mixture of both types of proteins. The failure to detect bands 7 and 8 on the electropherogram (o-dianisidine stained) prepared from the crude myoglobin is most probably the result of the low concentration of these components in the preparation.

The homogeneous myoglobin preparation (P3) was dialyzed against potassium chloride solution (0.1 N, 4°C)

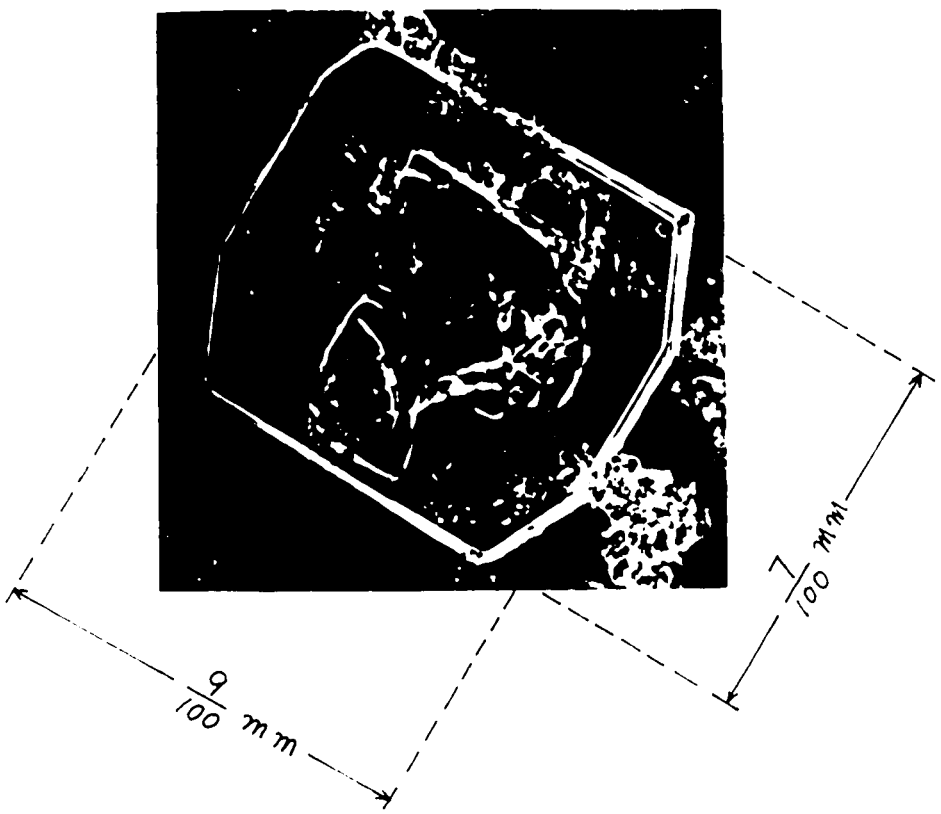
and was then dialyzed against glass distilled water (4°C). The solution was then freeze dried and stored over sodium hydroxide flakes (4°C). (This will be referred to as homogeneous myoglobin).

The homogeneous myoglobin was dissolved in water (30 mg/ml) and the resultant solution was then dialyzed against saturated ammonium sulfate solution (with excess ammonium sulfate, 4°C, pH 6.7) overnight. The myoglobin which precipitated was examined under a microscope and was found to be coarse but no definite shape was observed (Figure 18). The precipitate that was collected by centrifugation was dissolved in a small amount of distilled water and was again dialyzed against saturated ammonium sulfate solution (4°C, pH 6.7) overnight. The precipitate which still was not crystalline was suspended in the saturated ammonium sulfate solution and the suspension was allowed to stand in a cold room (4°C). A sample of the suspension was examined periodically and after one week crystals of myoglobin appeared as octagonal plates. Figure 18 shows a picture of a pork myoglobin crystal which was obtained by using an Ultraphotomicroscope (Karl Zeiss, Germany). The crystals resembled the pork myoglobin crystals which were obtained by Rossi and Travia (1941).

Figure 18. Crystal of Pork Myoglobin

(A) Coarse ppt without a definite shape

Figure 18. Crystal of Pork Myoglobin



(B) Octagonal plate

The homogeneous myoglobin preparation (P3) was used for the characterization of the protein.

4. CHARACTERIZATION OF PORK MYOGLOBIN

A. CHEMICAL ANALYSES

Pork myoglobin was analyzed for its moisture, ash, total nitrogen, and iron content. Table 4 shows the results of the analysis along with the results of the analysis of various myoglobins reported by other workers.

Bowen (1948) examined the iron content of horse myoglobin carefully by the method of Delory (1943). The author pointed out that the molecular weight of myoglobin calculated from the iron content of 0.323% is 17,291 while iron contents of 0.345% and 0.340% give values of 16,186 and 16,424, respectively. An iron content of 0.314% which was obtained for pork myoglobin gives a molecular weight of 17,787.

B. PARTIAL SPECIFIC VOLUME

The partial specific volume of pork myoglobin was calculated by use of the following equation (Gibbons, 1966):

$$\bar{V} = \frac{1}{\rho_s}\left(1 - \frac{\Delta\rho}{c}\right)$$

Table 4. Chemical Analysis of Pork Myoglobin

	Moisture %	Ash %	Nitrogen %	Iron %	
Pork myoglobin	7.8	0.53	16.67*	0.314*	
Horse myoglobin			16.65	0.345	Theorell (1932)
Human myoglobin				0.340	Theorell and de Duve (1947)
Human myoglobin			16.50	0.340	Rossi-Fanelli (1948)
Horse myoglobin				0.323	Bowen (1948)
Pork myoglobin			16.50	0.342	Rossi and Travia (1941)
Horse myoglobin			16.50	0.340	Rossi and Travia (1941)
Human myoglobin				0.318	Perkoff et al. (1962)
Finback whale myoglobin				0.308	Atassi and Saplin (1966)

*Corrected for moisture and ash content other than theoretical iron content of myoglobin.

Note: If it is assumed that the molecular weight of myoglobin is 18,000 and that there is one atom of iron per molecule of myoglobin, then the ash content (ferric oxide) of pure myoglobin would be 0.44%. If the pork myoglobin preparation is pure myoglobin contaminated with salts from the buffer, then the ash which would be produced by this contamination would be 0.53-0.44=0.09%. If the moisture content of pork myoglobin is 7.8%, then the amount of contamination would be 7.8+0.09%=7.89%. Hence the results of the nitrogen and iron analysis should be divided by a factor of 0.9211 to obtain the values on a salt and moisture free myoglobin.

where:

$\bar{V}$: partial specific volume

ρ_s: solvent density

$\Delta\rho$: difference in density between solution at concentration c (g/ml) and solvent

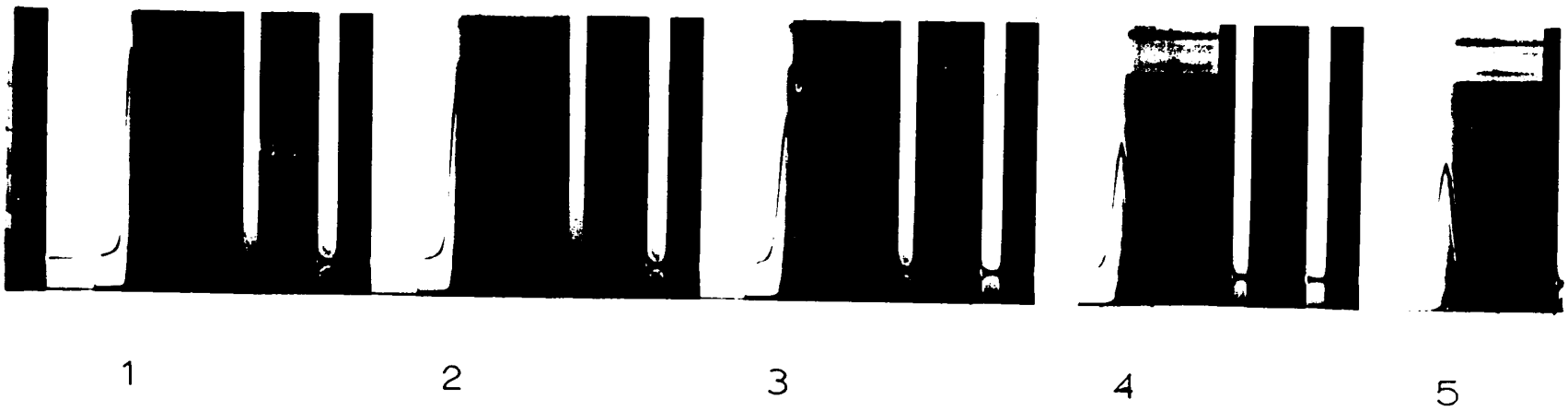
c: concentration of myoglobin solution (g/ml)

The density of a myoglobin solution (0.4%, 0.5%, 0.6% W/V) was measured by means of a pyknometer. The value which was obtained for the partial specific volume of pork myoglobin was 0.721. Perkoff et al. (1962) and Atassi and Saplin (1966) reported values for the partial specific volume of human and finback whale myoglobins of 0.743 and 0.724, respectively.

C. SEDIMENTATION COEFFICIENT

The sedimentation velocity of pork myoglobin was measured in a phosphate buffer (0.1 M, pH 7.0, 0.01% KCN, 25°C) by means of a Spinco Model E Ultracentrifuge (synthetic boundary cell, 59,780 rpm). Figure 19 shows pictures of the sedimentation pattern that were obtained during a measurement. The sedimentation coefficients that were obtained using myoglobin solutions of different concentrations (i.e. 0.3%, 0.5%, 1.1% W/V) were 1.94S, 1.92S and 1.90S (S: Svedberg 10^{-13} second or 10^{-13} cm/sec./dyne/g),

Figure 19. Pictures of sedimentation pattern of pork myoglobin



1 pattern at 8 minutes after reaching speed
2 pattern at 6 minutes after reaching speed
3 pattern at 24 minutes after reaching speed
4 pattern at 32 minutes after reaching speed
5 pattern at 40 minutes after reaching speed

respectively. The values were extrapolated to zero protein concentration to give a value of 1.95S.

Perkoff et al. (1962) and Atassi and Saplin (1966) reported values of 1.815S and 1.96S for human and finback whale myoglobins, respectively.

D. MOLECULAR WEIGHT

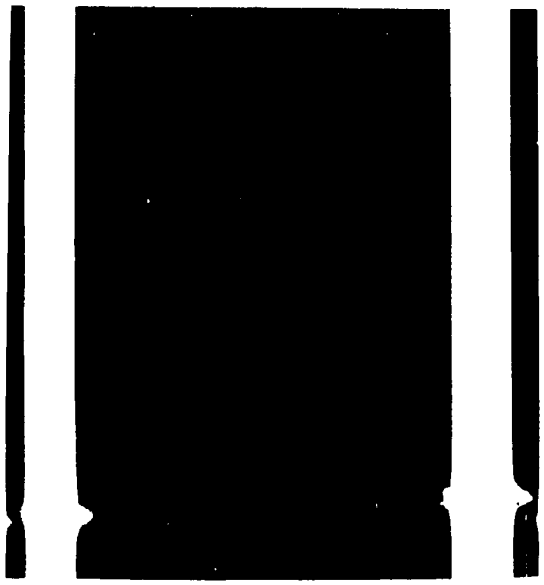
The molecular weight of pork myoglobin was determined from ultracentrifugation data using the method of Schachman (1957). An ultracentrifugal run (in a phosphate buffer 0.1 M, pH 7.0, containing 0.01% KCN, 25°C) was performed using a Spinco Model E Ultracentrifuge (standard cell, 10,589 rpm). Figure 20A shows a pattern which was obtained during the run. Figure 20B shows a schematic diagram which was used for the calculation of molecular weight from the run during the approach to equilibrium. The values for the molecular weight of pork myoglobin which were obtained were as follows:

Mb: (molecular weight calculated at the cell bottom	17,500
Mm: (molecular weight calculated at the meniscus)	18,400

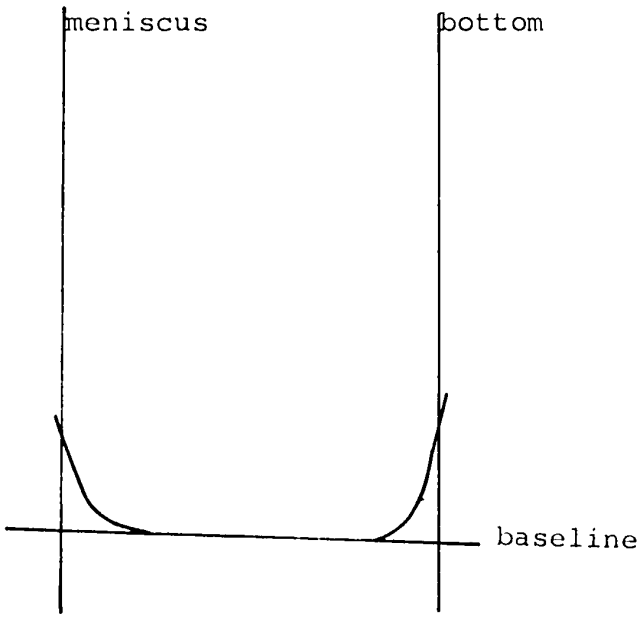
Perkoff et al. (1962) obtained a value of 17,900 for human myoglobin from sedimentation-diffusion measurements. Atassi and Saplin (1966) obtained values of 17,950 and 18,200 for weight average molecular weight and number

Figure 20. Pictures of a Run During the Approach to Equilibrium of Pork Myoglobin

(A) Picture of a pattern for the calculation of molecular weight from a run during the approach to equilibrium



(B) Schematic diagram for the calculation



average molecular weight, respectively, for finback whale. These authors used data obtained from sedimentation equilibrium measurements for the molecular weight determinations.

E. AMINO ACID ANALYSIS

The pork myoglobin hydrolyzates (II.11.) were analyzed for their amino acid content by use of a Beckman Spinco amino acid analyzer. A typical chromatogram is shown in Figure 21. The results of the analysis are given in Table 5. The values for aspartic acid, threonine, serine and lysine showed a linear decrease with time of hydrolysis. The average values for these amino acids were obtained by extrapolation to zero hydrolysis time (Smith and Stockell, 1954). The extrapolations are illustrated in Figure 22. The values for valine, isoleucine, leucine, and phenylalanine which are reported represent the average of two values obtained with the 72-hour hydrolyzate and two obtained with the 96-hour hydrolyzate. Methionine sulfoxide peaks were noted on the chromatogram. As a sulfoxide standard was not available, the methionine content was estimated from the pooled peak area of methionine and methionine sulfoxide peaks.

Tryptophan was determined by the spectrophotometric

Figure 21. Chromatogram of Amino Acid Analysis by a Beckman Spinco Amino Acid Analyzer 120 C

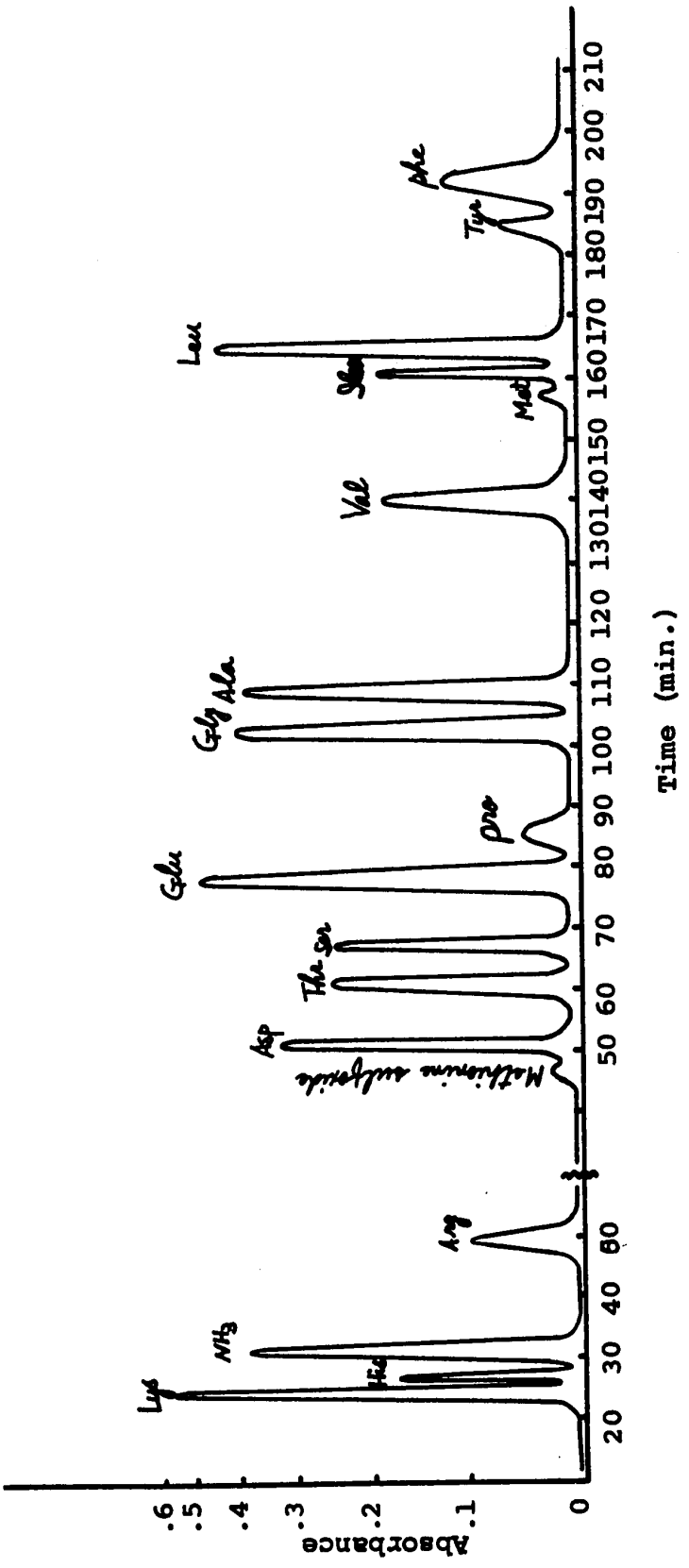


Table 5. Amino Acid Analysis of Hydrolysates of Pork Myoglobin

	Gram amino acid per 16 g Nitrogen[1]					Gram amino acid per 16.67 g Nitrogen	Amino acid residue/ mole	Nearest integer	Molecular weight
	Time of hydrolysis (hr)				Average				
	24	48	72	96					
Aspartic acid	8.15	7.55	7.05	6.65	8.60[2]	8.94	13.05	13	1366.3
Threonine	3.92	3.74	3.45	3.17	4.20[2]	4.39	6.63	7	707.7
Serine	2.43	2.25	2.16	2.02	2.60[2]	2.70	4.61	5	435.5
Glutamic acid	14.95	14.54	14.79	14.32	14.65	15.24	18.59	19	2452.9
Proline	2.87	2.57	2.73	2.31	2.62	2.72	4.25	4	388.4
Glycine	4.12	4.04	4.07	3.98	4.05	4.21	10.12	10	571.0
Alanine	8.39	7.76	8.12	7.61	7.97	8.29	16.74	17	1208.7
Valine	(4.81)	(4.51)	5.27	5.11	5.19[3]	5.40	8.32	8	792.8
Methionine	(0.70)[4]	(0.93)[4]	1.04[4]	1.06[4]	1.05[3]	1.10	1.34	1	131.2
Isoleucine	(3.65)	(4.73)	5.81	5.57	5.69[3]	5.92	8.11	8	905.6
Leucine	(9.09)	(9.66)	10.94	11.12	11.03[3]	11.47	15.71	16	1811.2
Tyrosine	2.20	2.04	2.11	1.97	2.08	2.16	2.14	2	326.4
Phenylalanine	(3.84)	(4.24)	5.02	4.94	4.98[3]	5.18	5.65	6	883.2
Lysine	15.35	15.21	14.91	14.58	15.74[2]	16.37	20.14	20	2564.0
Histidine	8.65	8.34	8.62	8.18	8.44	8.78	10.19	10	1372.0
Arginine	3.21	3.02	3.11	2.93	3.07	3.19	3.29	3	468.6
Tryptophan							1.95[5]	2	372.4
Sub total						106.60		151	16757.9
Heme									488
TOTAL									17245.9

[1]Average of two determinations

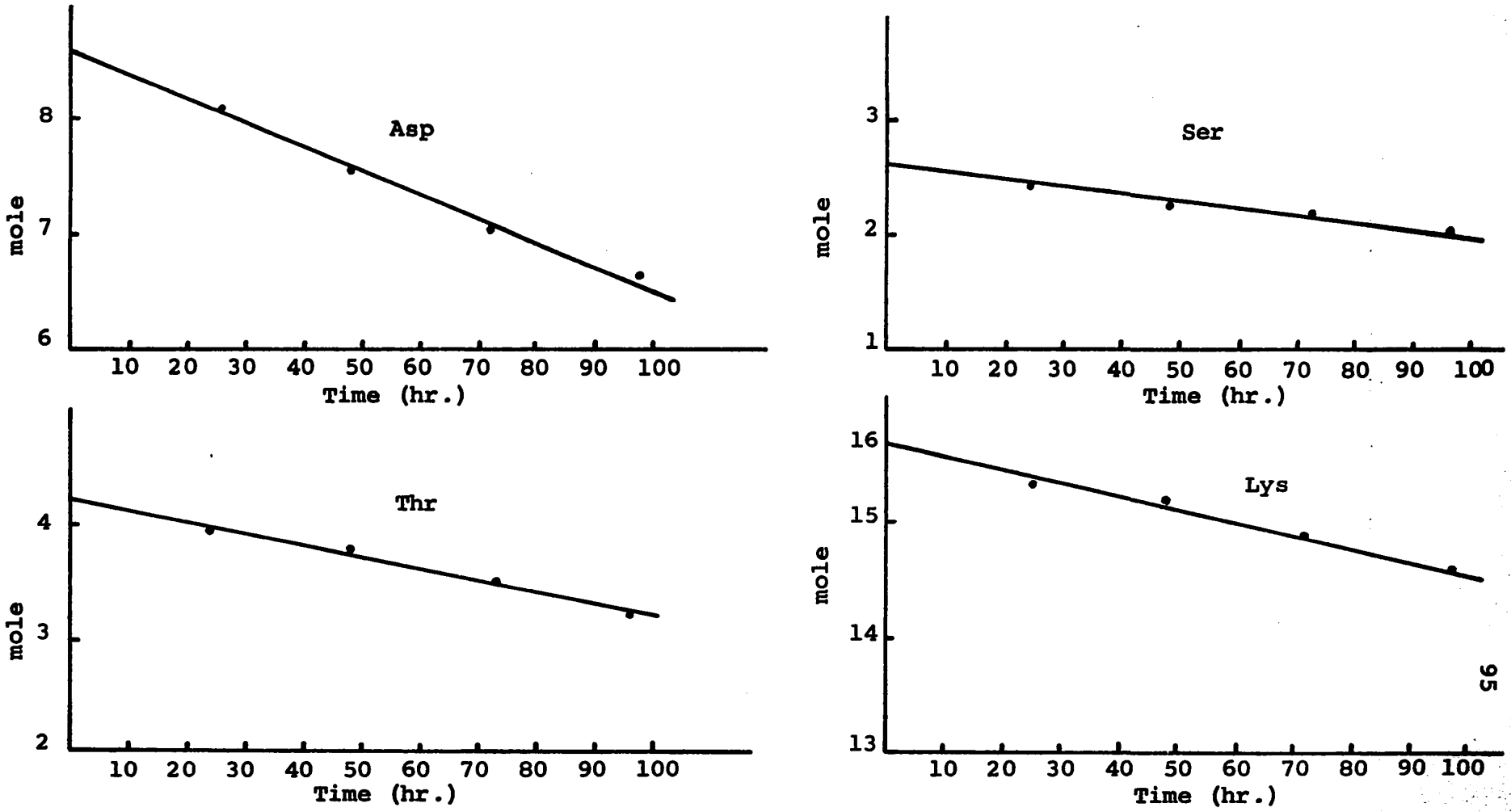
[2]Values obtained by extrapolation to zero hydrolysis time

[3]The averages do not include the values in parentheses

[4]Values corrected for the formation of sulfoxides

[5]Value obtained by spectrophotometric method

Figure 22. Extrapolation of amino acids to zero hydrolysis time



method of Bencze and Schmid (1957). Solutions were prepared containing different concentrations of tryptophan and tyrosine. The absorption spectra (250 to 300 mμ) of the solutions were measured by means of a Spectronic 505 spectrophotometer. The readings were plotted against wave length. Figure 23 shows the absorption spectra which were obtained with the solution having a tyrosine-tryptophan molar ratio (R) of 0.5. A line was drawn tangent to the two peaks (line AB, Figure 23). The value for the slope of this line (a/b) was obtained by dividing the absorbance intersection (a) by the wave length intersection (b). The a/b ratio was then divided by the maximum absorption (A max.) of the absorption curve and the resultant fraction was multiplied by 10^3 to give S. Table 6 lists the values of S and the extinction coefficients obtained for the solutions with different R values.

Two samples (2.7 mg, 2.5 mg) of pork myoglobin were each dissolved in sodium hydroxide solution (5 ml, 0.1 N) and the absorption spectra (250 to 300 mμ) of each solution were measured. An S value of -2.8 was obtained for each of the two solutions. It will be noted from the table that an S value of -2.8 corresponds to a tyrosine-tryptophan molar ratio of 1.0. From the corresponding extinction coefficient

Figure 23. Absorption Spectra of Tyrosine-Tryptophan

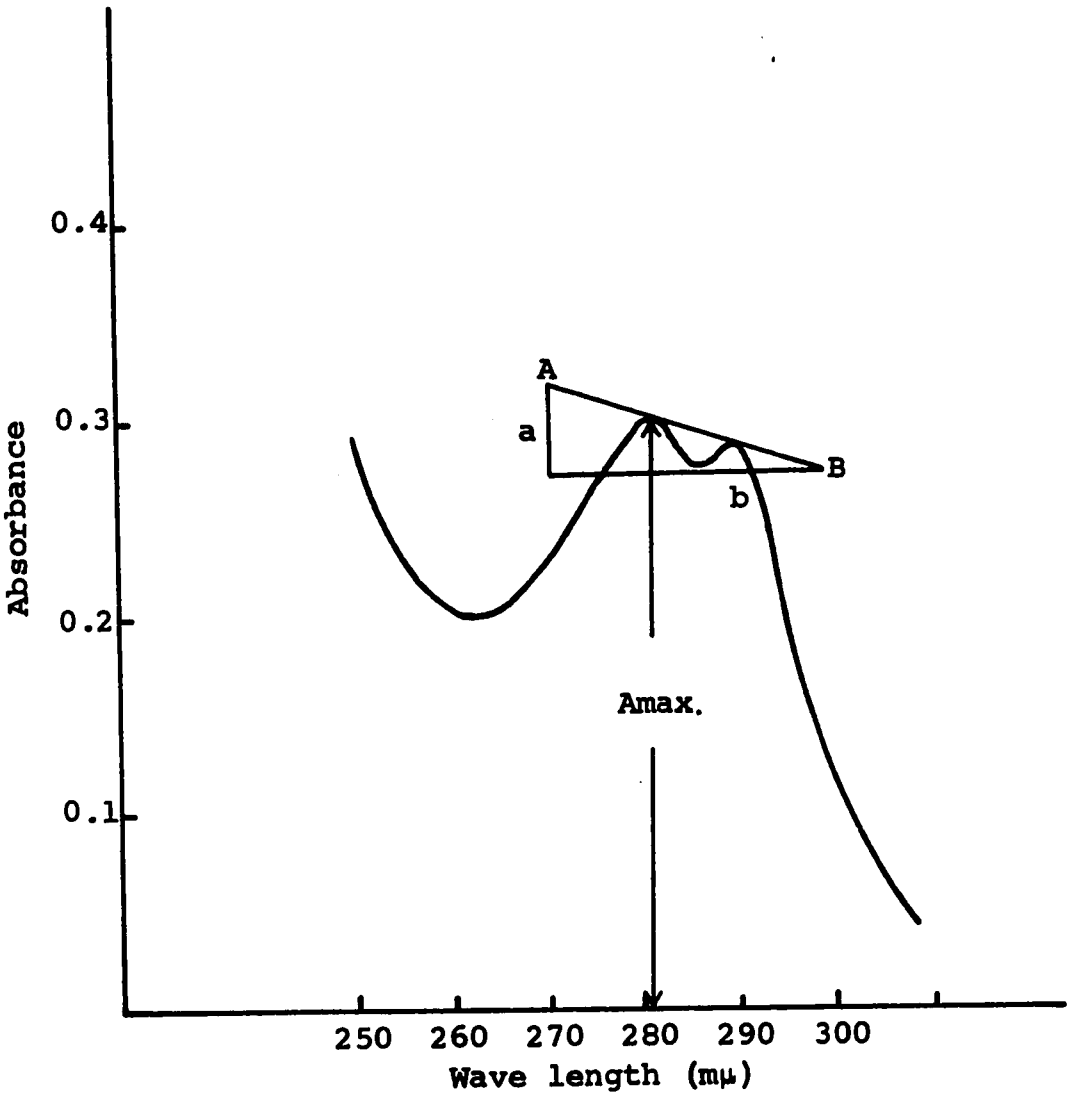


Table 6. Extinction Coefficient of Tyrosine-Tryptophan Mixture

	Molar Ratio Tyrosine/Tryptophan R	Extinction Coefficient $E^{1\%}_{1\,cm}$	S*
Tryptophan		269	
	0.1	253	-17.5
	0.3	230	-12.5
	0.5	215	- 8.9
	0.9	193	- 3.9
	1.0	191	- 2.8
	1.1	189	- 1.8
	1.5	178	+ 1.5
	2.5	165	+ 8.5
	4.0	153	+18.5
	6.0	148	
	20.0	138	
Tyrosine		135	

* $S = \frac{(a/b) \times 10^3}{A \text{ max.}}$

(191, Table 6) the content of tyrosine and tryptophan in the sample was found to be 3.78%. If a value of 18,000 is used for the molecular weight of myoglobin, the tryptophan and

tyrosine content of pork myoglobin may be calculated as follows:

$$\frac{3.78}{100} \times 18{,}000 \times \frac{1}{(204-18) + (181-18)} = 1.95 \text{ (moles)}$$

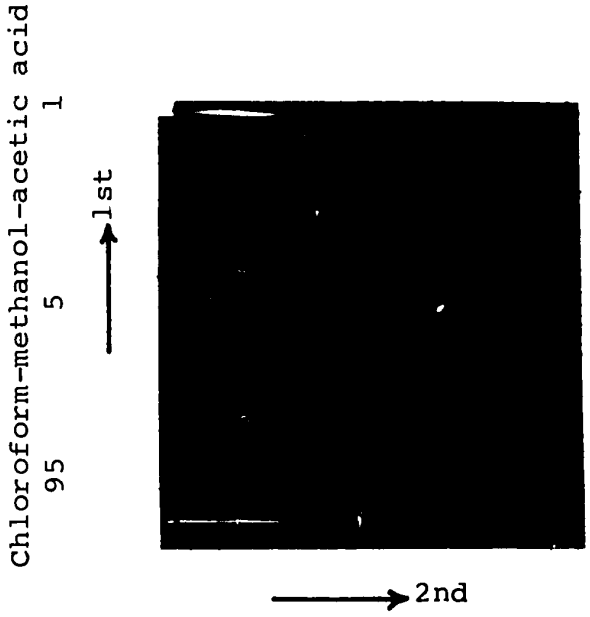
The number of moles of tyrosine as determined by this method (1.95 moles) is in good agreement with the number of moles of tyrosine (2.14) as determined by the Moore and Stein method (II.11.). The result shows that the tryptophan content of pork myoglobin is two moles tryptophan per mole protein.

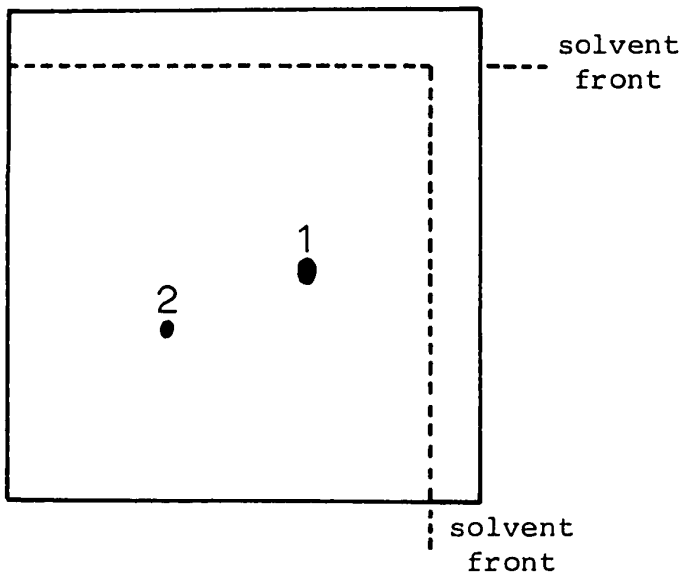
F. IDENTIFICATION OF AMINO TERMINAL RESIDUE OF PORK MYOGLOBIN

a) By use of 2-chloro-3,5-dinitropyridine

Pork myoglobin (30 mg) was treated with 2-chloro-3,5-dinitropyridine and the protein derivative was hydrolyzed with hydrochloric acid. The dinitropyridyl amino acid derivatives were extracted with ethyl acetate and were analyzed by thin-layer chromatography. Figure 24 shows the picture of a two dimensional thin-layer chromatogram. Two spots were observed with spot 1 as the major spot. The R_f values of the spots were compared with those of standard dinitropyridyl amino acids (alanine, aspartic acid, glutamic acid, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, valine,

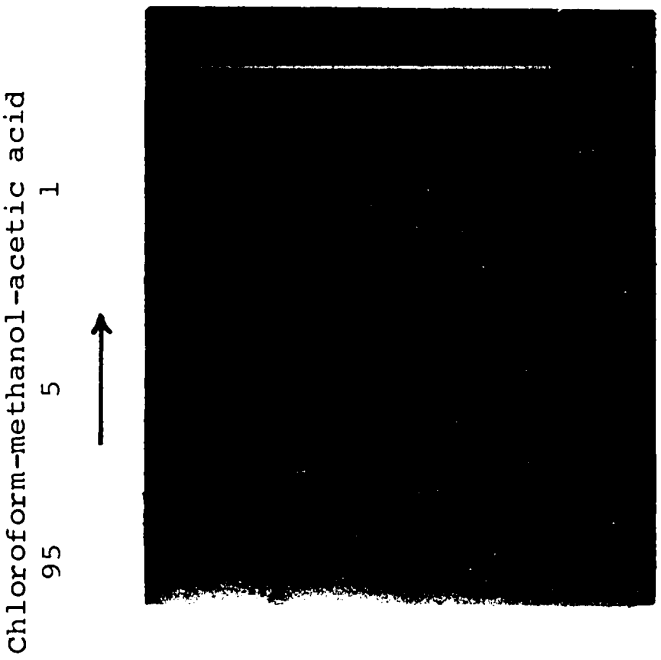
Figure 24. Thin-Layer Chromatogram of Dinitropyridyl Amino Acid Derivative

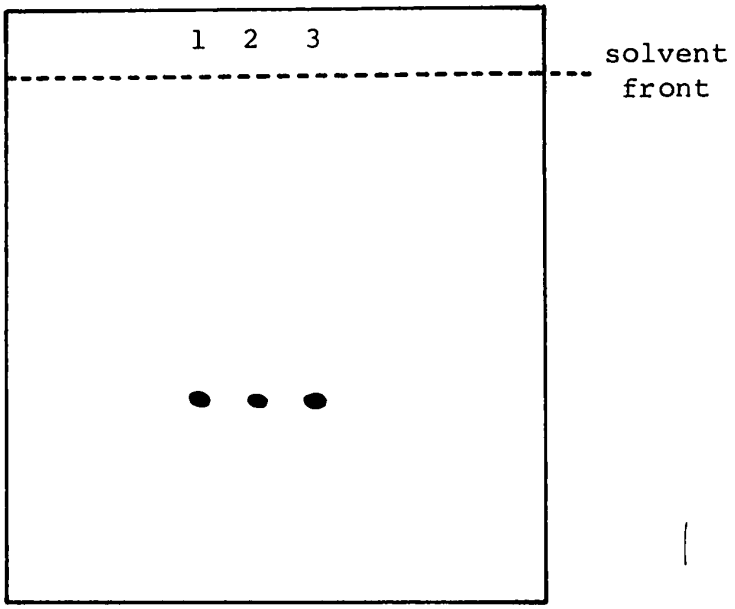




(A) Two dimensional

Figure 24.





1 and 3 dinitropyridyl valine
2 spot 1

(B) One dimensional

histidine, lysine, tyrosine, arginine, and hydroxyproline). The results indicate that spot 1 is the dinitropyridyl derivative of valine. The addition of a small amount of standard dinitropyridyl valine to the ethyl acetate extract resulted in an enhancement of the intensity of spot 1 in the chromatogram similarly prepared. Spot 1 was scraped from the thin-layer chromatogram, and the dinitropyridyl derivative was eluted by use of ethyl acetate for further identification.

Identification of spot 1 was carried out by one dimensional thin-layer chromatography with standard dinitropyridyl valine alongside using four different solvent systems.

The four solvents used are as follows (Stahl, 1965):

1) Toluene system:

   Toluene-Pyridine-Ethylene chlorohydrin-0.8N ammonia solution (100:30:60:60 V/V)

2) Chloroform-Benzyl alcohol-Acetic acid (70:30:3 V/V)

3) Benzene-Pyridine-Acetic acid (80:20:2 V/V)

4) Chloroform-Methanol-Acetic acid (95:5:1 V/V)

The identification of spot 1 was also performed using commercially available dinitropyridyl valine (Nutritional Biochemicals Corporation). A typical one dimensional thin-layer chromatogram is shown in Figure 24. The results

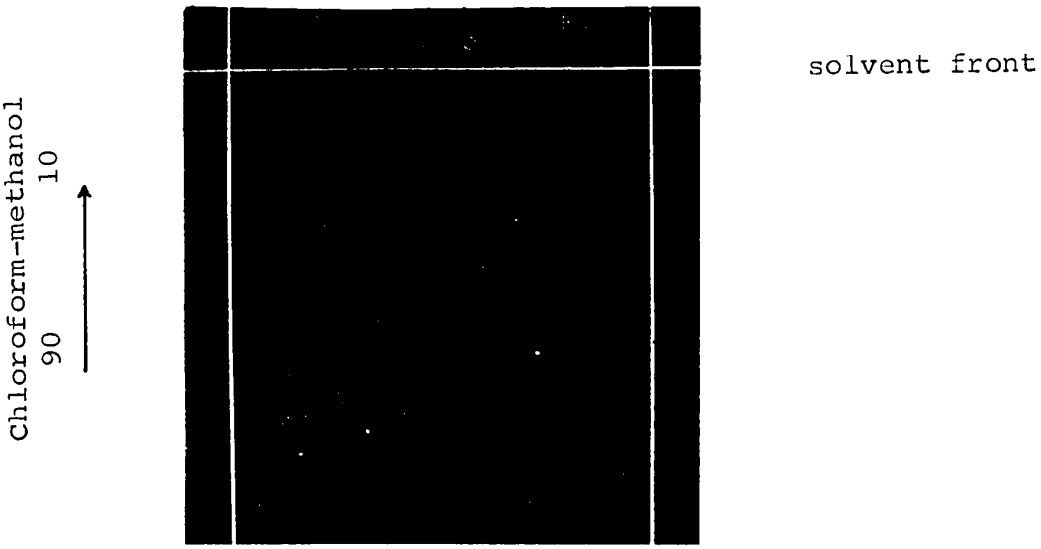
showed that spot 1 is dinitropyridyl valine.

The author was unable to identify spot 2 with any of the available standard dinitropyridyl amino acid derivatives.

b) By use of phenylisothiocyanate

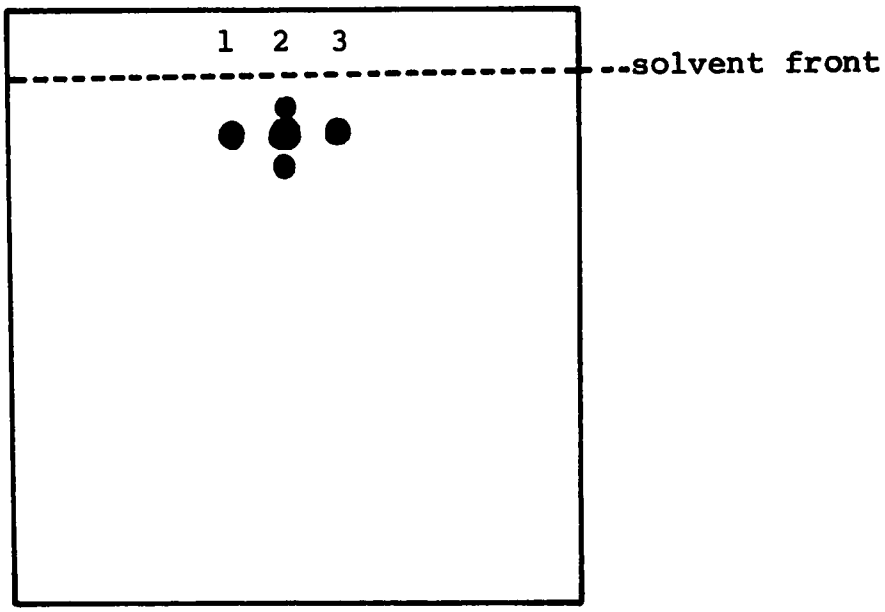
Pork myoglobin (30 mg) was treated with phenylisothiocyanate and the protein derivative was degraded to liberate phenylthiohydantoins. The phenylthiohydantoin derivatives were analyzed by thin-layer chromatography and phenylthiohydantoin valine was used as a standard. Figure 25 shows a thin-layer chromatogram which was obtained using the solvent system chloroform-methanol (90:10). Three spots were noted on the chromatogram. Spot No. 2 which was the major spot corresponded to phenylthiohydantoin valine. The analysis was repeated using chloroform as solvent. Phenylthiohydantoin valine was again identified as the major component. No attempt was made to identify the two minor spots as the major spot was identified as phenylthiohydantoin valine and this agreed with the results of the amino terminal analysis by the use of 2-chloro-3,5-dinitropyridine. Atassi and Saplin (1966) carried out the Edman degradation of finback whale myoglobin and identified the phenylthiohydantoin derivative of five amino acids in the first cyclization step. It was concluded that valine is the amino terminal residue of pork myoglobin.

Figure 25. Thin-Layer Chromatogram of Phenylthiohydantoin Derivative of Amino Acid



1 and 3 phenylthiohydantoin valine
2 phenylthiohydantoin derivatives liberated from pork myoglobin

Figure 25.



Edmundson and Hirs (1961) and Atassi and Saplin (1966) reported valine as the amino-terminal residue of sperm whale and finback whale myoglobin, respectively. Akeson and Theorell (1960), Rumen (1959), Perkoff et al. (1962) and Hapner et al. (1968) reported glycine as amino-terminal residue of horse, seal, human and harbor seal and porpoise myoglobins, respectively.

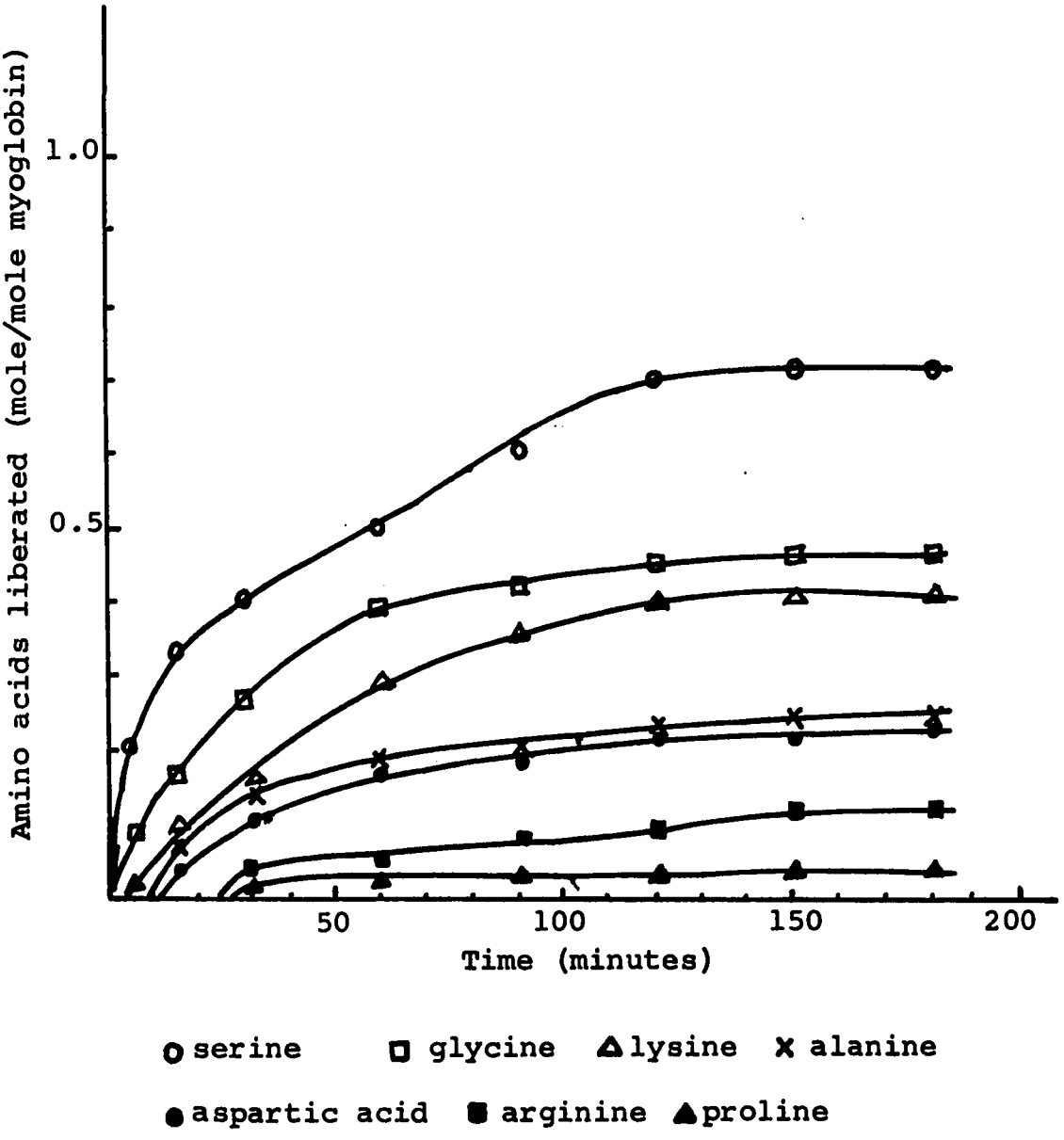
G. CARBOXYL TERMINAL RESIDUES OF PORK MYOGLOBIN

Pork myoglobin (40 mg) was treated with carboxypeptidase (Nutritional Biochemicals Corporation) and the amino acids which were liberated were analyzed by the use of a Beckman Spinco amino acid analyzer. The amount of amino acids liberated was plotted against time of treatment with carboxypeptidase. Figure 26 shows the rate of release of amino acids from pork myoglobin by the action of carboxypeptidase.

The results showed that the amino acids were liberated in the order of serine, glycine, lysine, alanine, aspartic acid, arginine, proline. The result suggests that the carboxyl terminal residue of pork myoglobin is serine.

Other workers have reported the carboxy-terminal amino acid sequence of horse, human, sperm whale and

Figure 26. The Rate of Release of Amino Acids by Carboxypeptidase CT from Pork Myoglobin



finback whale myoglobins.

Horse myoglobin (Akeson and Theorell, 1960):

-leucine-glycine-tyrosine-glycine-glutamine (carboxy-terminal)

Human myoglobin (Perkoff et al., 1962):

-leucine-glycine-phenylalanine-glycine-glutamine (carboxy-terminal)

Sperm whale myoglobin (Edmundson and Hirs, 1962b):

-glutamic acid-leucine-glycine-tyrosine-glycine-glutamine (carboxy-terminal)

Finback whale myoglobin (Atassi and Saplin, 1966):

-serine>glycine>lysine>alanine>aspartic acid

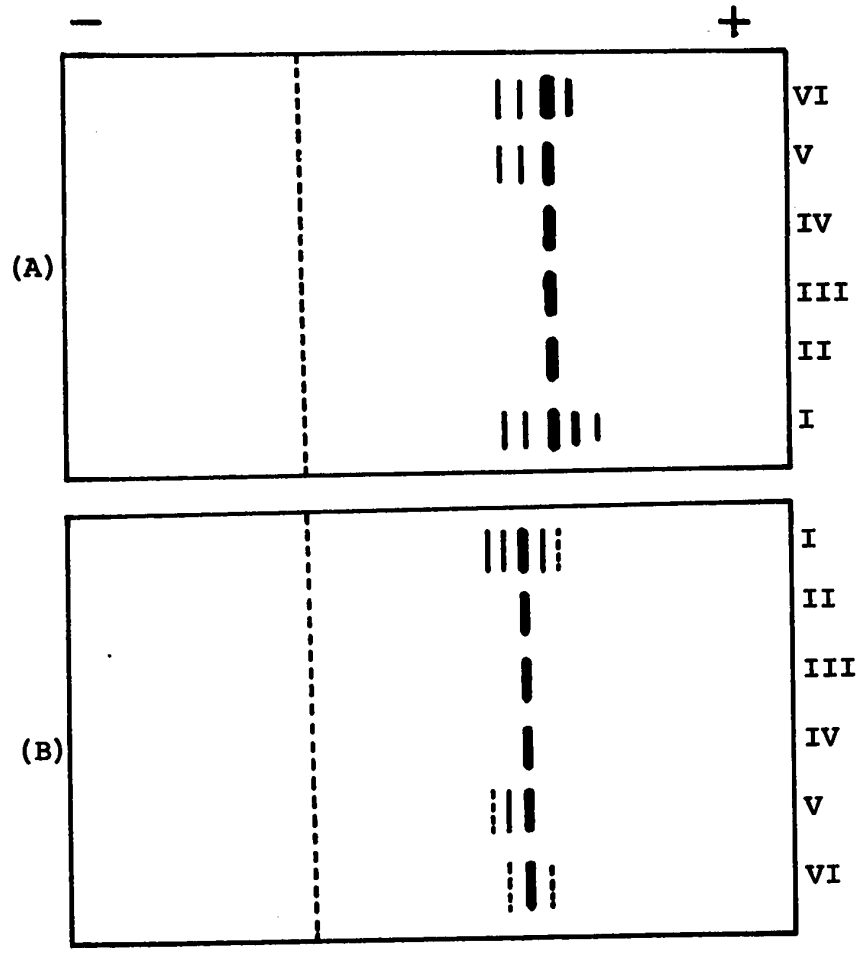
H. EFFECTS OF TEMPERATURE, DIALYSIS AND FREEZE-DRYING ON PORK MYOGLOBIN

A homogeneous myoglobin preparation was subjected to various treatments and was then examined for homogeneity by starch-gel electrophoresis. Figure 27 shows that storage of myoglobin solution for 24 hours at room temperature or dialysis of myoglobin solution against distilled water did not alter the homogeneity of the myoglobin. Freeze-drying, however, changed the homogeneous myoglobin into a mixture of two protein components.

5. GENERAL DISCUSSION

Morgan (1936) and Kendrew et al. (1954) suggested

Figure 27. Electropherogram of Physically Treated Pork Myoglobins (Schematic representation)



(A) o-dianisidine stained
(B) amido black 10B stained

I crude pork myoglobin
II homogeneous pork myoglobin (P3, Figure 17)
III P3 exposed to room temperature for 24 hours
IV P3 dialyzed
V P3 freeze-dried
VI P3 dialyzed and then freeze-dried

Note: Gel B shrank due to the organic solvent wash. This accounts for the non-alignment of the bands in the two gels.

chat the difficulties they encountered in their attempts to crystallize the myoglobins of some of the common domestic animals as compared to the relative ease of crystallization of the myoglobins of other animals, might be due to species differences in the myoglobins. Roche et al. (1942) and Lewis and Schweigert (1955) were unsuccessful in their attempts to prepare crystalline pork myoglobin. Preliminary attempts in our laboratory to prepare crystalline myoglobin from a salt precipitated myoglobin preparation (Fraction 8) were also unsuccessful. The difficulty of crystallizing pork myoglobin could be due to the presence in pork muscle of several proteins which have solubility characteristics that are similar to those of myoglobin. For example, experiments showed that the myoglobin which was prepared by the conventional salt precipitation method comprises three non-heme proteins (Figure 14, bands 6, 7, 8) and five heme proteins (i.e. myoglobins, bands 1, 2, 3, 4, 5).

A review of the literature revealed that Amberlite IR-50 resin (Boardman and Adair, 1956; Edmundson and Hirs, 1961), DEAE-cellulose (Perkoff et al., 1962) and CM-cellulose (Rumen, 1959; Atassi and Saplin, 1966; Hardman et al., 1966) have been used for the fractionation of the myoglobins of

a number of different species. The results of these workers showed that CM-cellulose gave the most satisfactory fractionation. Perkoff et al. (1962) fractionated human myoglobin on DEAE-cellulose and showed that each fraction comprised two or three electrophoretic (starch-gel) components. However, when the myoglobins of the various fractions were converted to cyanmetmyoglobins and then subjected to starch-gel electrophoresis, only one electrophoretic component was detected in each fraction. Perkoff et al. (1962) reasoned that the difference in the electrophoretic behavior of the myoglobin was the result of the difference in the state of the heme prosthetic group. Hence conversion of metmyoglobin into cyanmetmyoglobin would eliminate the difference in the heme prosthetic group. Atassi and Saplin (1966) fractionated finback whale myoglobin (cyanmetmyoglobin) into nine fractions using CM-cellulose and obtained three homogeneous preparations. In the present work the author has attempted to fractionate by use of CM-cellulose, the pork myoglobin which was obtained by salt precipitation after conversion of the metmyoglobin to cyanmetmyoglobin.

Rumen (1959), Edmundson and Hirs (1962), Perkoff et al. (1962), and Atassi and Saplin (1966) showed that

the amino acid composition of the major component of a myoglobin mixture which was obtained from a given species, was identical to those of the minor myoglobin components from the same species. The chromatographic (CM-cellulose) method that was used in the present work for the fractionation of pork myoglobin was designed to separate the major component (band 3, Figure 14) from the minor components (bands 1, 2, 4, 5, Figure 14) and also from the non-heme proteins (bands 6, 7, 8, Figure 14). The method is similar to the methods reported by previous workers and especially to the method of Atassi and Saplin (1966). Figure 16 illustrates the separation by this method of the major myoglobin component (P3) from the minor myoglobin components and also from the non-heme proteins. Approximately 50 hours were required for the method to isolate the major myoglobin component. Atassi and Saplin (1966) isolated the major myoglobin component and two minor myoglobin components in a chromatographic run which lasted for 300 hours.

The pork myoglobin which was prepared by salt precipitation contained eight electrophoretic components (Figure 14), five of which were heme proteins and three were non-heme proteins. Electrophoretic analysis (Figure 17) of the various fractions that were obtained by chromatographic (CM-cellulose) fractionation of the same myoglobin

preparation, showed the presence of seven heme proteins and six non-heme proteins. The greater number of electrophoretic components in the protein fractions was probably the result of the circumstance that certain protein components were present in such low concentration in the unfractionated preparation that they were not detected on the electropherograms.

Previous workers have shown that the crystalline myoglobins which have been prepared so far were electrophoretically heterogeneous. Perkoff et al. (1962) attributed this heterogeneity to the difference in the state of the iron in the heme prosthetic group. They showed that heterogeneous myoglobin preparations became homogeneous in the presence of cyanide. Edmundson (1965) suggested that the heterogeneity of crystalline myoglobin might be the result of hydrolysis of glutamine and asparagine to glutamic and aspartic acid residues. Atassi and Saplin (1966) demonstrated that the number of electrophoretic components in a myoglobin preparation changed when the product was subjected to such treatments as dialysis and freeze-drying. Similar observations were made on pork myoglobin when it was freeze-dried.

The absorption maxima of pork myoglobin, as shown in

Table 2, in its different forms (i.e. oxymyoglobin, metmyoglobin and cyanmetmyoglobin) are similar to those of whale protomyoglobin (Smith and Gibson, 1959) and human myoglobin (Perkoff et al., 1962). These results suggest that the porphyrin group of pork myoglobin is also a protoporphyrin (Figure 1).

Table 7 shows the partial specific volume, sedimentation coefficient and molecular weight of pork myoglobin along with those of human (Perkoff et al., 1962) and finback whale myoglobin (Atassi and Saplin, 1966).

Table 7. Physical properties of myoglobins

	PORK	HUMAN (Perkoff et al., 1962	FINBACK WHALE (Atassi and Saplin, 1966)
Partial specific volume	0.721	0.743	0.724
Sedimentation coefficients	1.95 S(25°C)	1.815 S(20°C)	1.96 S(20°C)
Molecular weight	Mb=17500	17900	17950 (weight average)
	Mm=18400		18200 (number average)

Physical properties of pork myoglobin which was prepared in this laboratory are similar to those of other myoglobins prepared by previous workers. However, it is well known

that the values obtained for the physical characteristics may vary slightly depending on the method and technique employed in the determination.

Amino acid analysis of pork myoglobin showed that pork myoglobin like other mammalian myoglobins (Timmer et al., 1957; Akeson and Theorell, 1960; Rumen, 1960; Perkoff et al., 1962; Edmundson and Hirs, 1962a; Atassi and Saplin 1966; Hapner et al., 1968) contains no cysteine. It is interesting to note that fish myoglobins (Konosu et al., 1958; Hirs and Olcott, 1964) contain one residue of cysteine per mole of myoglobin.

Calculation based on the amino acid composition of pork myoglobin showed that each molecule of pork myoglobin comprises 151 amino acid residues. Table 8 tabulates the amino acid composition of the myoglobins of various species. The results illustrated that the number of amino acid residue per mole of myoglobin ranges from 146 to 155. The observed difference in the number of amino acid residue may be due to the number of factors including (a) species difference in myoglobins, (b) experimental error involved in the determination of amino acid and (c) degree of purity of myoglobin preparation used in the investigation.

Table 8. Amino Acid Composition of Myoglobins from Various Species

Amino acid	Amino Acid Composition of Myoglobin (residues/mole)												
	Ox[1]	Sheep[1]	Horse[1]	Seal[1]	Human[1]	Dorphin[1]	Sperm whale[1]	Finback whale[1]	Harbor seal[2]	Porpoise	Pig[3]	Blue -fin tuna[1]	Yellow -fin tuna[1]
Asp	14	12	11	11	12	12	8	12	11	10	13	12	12
Thr	5	5	7	5	4	5	5	5	5	6	7	7	7
Ser	5	5	5	7	8	5-6	6	5	7	5	5	4	5
Glu	17	16	19	16	21	17	19	17	17	18	19	11	11
Pro	4	3	4	4	6	4	4	4	4	4	4	6	5
Gly	12	14	16	12	15	14	11	11	12	14	10	14	14
Ala	16	17	16	14	11	18	17	18	14	15	17	21	21
Cys (half)	0	0	0	0	0	0	0	0	0	0	0	1	1
Val	9	9	7	6	8	6	8	6	6	6	8	9	8
Met	2	3	2	2	3	2	2	2	2	2	1	2	3
Ile	5	6	9	7	7	8	9	9	8	8	8	9	10
Leu	18	19	18	18	16	20	18	17	19	19	16	16	17
Tyr	2	2	2	2	2	2	3	2	2	2	2	2	2
Phe	6	9	7	7	7	8	6	7	7	7	6	6	6
Lys	19	19	19	18	19	21	19	20	19	20	20	15	15
His	13	11	11	12	9	12	12	11	13	12	10	5	6
Arg	3	2	2	5	3	3	4	3	5	3	3	2	2
Try	ND	ND	ND	ND	3	2	2	2	2	2	2	1	ND
Total	150	152	155	146	154	147-148	153	151	153	153	151	143	145

ND Not determined

[1] Quoted from Atassi and Saplin (1966)

[2] Quoted from Hapner et al. (1968)

[3] This thesis

It is interesting to note that the myoglobins of tunas contain about half as much histidine as do the mammalian myoglobin and that they contain one residue of cystein per mole. The amino acid compositions of the various mammalian myoglobin are fairly similar. Atassi (1966) calculated the mole percent (number of residue x 100/total number of residue in protein) value (N) for the following amino acid group: serine + threonine (Nst); alanine + valine + isoleucine + leucine (Navil); arginine + histidine + lysine (Nahl). Since phenylalanine showed appreciably less fluctuation in myoglobin than did alanine he also calculated the value phenylalanine + valine + isoleucine + leucine (Npvil). In this way the author was able to point out a constant and characteristic composition tendencies that were typical of a particular class in the evolution ladder. Table 9 lists the mean of the N values for mammalian myoglobin along with the corresponding value for pork myoglobin.

Table 9. Amino acid Content of Mammalian Myoglobin

	Mammalian myoglobin	Pork myoglobin
Nst	7.31 $\pm$ 0.81	7.94
Navil	30.63 $\pm$ 3.37	32.45
Npvil	26.54 $\pm$ 1.54	25.16
Nahl	22.15 + 1.78	21.85

It will be noted that the N values for pork myoglobin fall within the range of the N values of the mammalian myoglobin.

Valine was found to be the amino-terminal residue of pork myoglobin. It may be of interest to note that sperm whale myoglobin (Edmundson and Hirs, 1961) and finback whale myoglobin (Atassi and Saplin, 1966) also have valine as their amino-terminal residue whereas seal (Rumen, 1959), horse (Akeson and Theorell, 1960), human (Perkoff et al., 1962), harbor seal and porpoise (Hapner et al., 1968) have glycine as the amino-terminal residue.

IV. SUMMARY

1) A water extract of pork muscle was fractionated by acetone precipitation and column chromatography (CM-cellulose, Sephadex-G 75) according to the method of Scopes for the isolation of alkaline protein. A fraction (Fraction A) was isolated which was homogeneous as measured by starch-gel electrophoresis and by ultracentrifugation. The fraction contained 13.36% nitrogen, 13.53% hexose, 0.6 mg/g boron and gave a single absorption peak at 265 mμ over the range 220 to 700 mμ. It had an electrophoretic mobility that was similar to those of myoglobin and apo-myoglobin. Thin-layer and paper chromatographic analyses of a hydrolyzate of Fraction A showed that glucose was the only sugar present. On the basis of the composition, electrophoretic and ultracentrifugal behavior, and spectral analysis it was suggested that Fraction A might be a complex of apo-myoglobin, borate and a polyglucose unit.

2) An attempt was made to isolate myoglobin from a water extract of pork skeletal muscle, by salt precipitation. Spectral analyses of products at different stages of preparation showed that the oxymyoglobin which was

originally present in the water extract, slowly changed to metmyoglobin during the isolation process. Starch-gel-electrophoretic analysis of the crude myoglobin that was obtained, showed that the preparation comprised at least five heme proteins and at least three non-heme proteins. Attempts to prepare crystalline myoglobin from the crude myoglobin preparation were unsuccessful.

3) The myoglobin which was present in the crude preparation, was converted to cyanmetmyoglobin and was then subjected to fractionation by use of CM-cellulose column chromatography. Five fractions were obtained only one of which was homogeneous as measured by starch-gel electrophoresis and the ultracentrifugation. This fraction (P3) represented 43% of the crude myoglobin preparation from which it was isolated.

4) The homogeneous myoglobin preparation (P3) was subjected to ultracentrifugal analysis and the following results were obtained:

sedimentation coefficient = 1.95S

molecular weight = 17,500 (Mb);
18,400 (Mm)

5) Calculation based on the iron (0.314%) contents of

the homogeneous myoglobin preparation (P3) showed that the minimal molecular weight of the pork myoglobin is 17,787.

6) The homogeneous myoglobin preparation (P3) was dissolved in water and then dialyzed (4°C) against concentrated ammonium sulfate solution. The protein precipitate that was obtained was checked periodically for the presence of crystalline myoglobin. After one week octagonal plates were observed and were photographed.

7) The amino acid composition of the homogeneous myoglobin preparation (P3) was determined by the use of a Beckman Spinco Amino Acid Analyzer. Tryptophan was determined by a spectrophotometric method. Calculation based on the amino acid composition of the myoglobin showed the presence of 151 amino acid residues per molecule of myoglobin and a molecular weight of 17,246.

8) The homogeneous myoglobin preparation (P3) was analyzed for amino-terminal residues by the use of 2-chloro-3,5-dinitropyridine and by the Edman degradation method. The results indicated that valine was the amino-terminal amino acid residue.

9) The homogeneous myoglobin preparation (P3) was

treated with carboxypeptidase. Aliquots of the digest were withdrawn at time intervals and were analyzed for their amino acid content. The results showed that serine was liberated in highest concentration followed by glycine, lysine, alanine, aspartic acid, arginine and proline.

V. CLAIMS TO ORIGINAL RESEARCH

1) The provision of data which suggest that a protein-carbohydrate complex may be formed during the acetone precipitation and chromatographic fractionation of the sarcoplasmic extract of pork skeletal muscle in buffer solutions containing borate ion.

2) The development of a method for the isolation of homogeneous pork myoglobin as determined by starch-gel electrophoresis and by ultracentrifugation.

3) The preparation of crystalline pork myoglobin from skeletal muscle.

4) The determination of the partial specific volume, the sedimentation coefficient and the molecular weight of pork myoglobin.

5) The determination of the amino acid composition of pork myoglobin and an estimation of the number of amino acid residue per mole of pork myoglobin.

6) The identification of the amino-terminal amino acid residue of pork myoglobin.

7) The provision of quantitative data on the liberation of amino acids from pork myoglobin by the action of carboxypeptidase.

REFERENCES CITED

Akeson, A., and Theorell, H.
On the microheterogeneity of horse myoglobin.
Arch. Biochem. Biophys. 91: 319 (1960).

Anastassiadis, P.A., Maw, W.A. and Common, R.H.
Studies on the glycoproteins of the domestic fowl. II. The hexosamine content of certain tissue of the sexually immature pullet and some effects thereon on gonadal hormones.
Can. J. Biochm. Physiol. 33: 627 (1955).

Anson, M.L. and Mirsky, A.E.
On some general properties of proteins.
J. Gen. Physiol. 9: 169 (1925).

Anson, M.L. and Mirsky, A.E.
Protein coagulation and its reversal. The preparation of insoluble globin, soluble globin and heme.
J. Gen. Physiol. 13: 469 (1930).

Antonini, E. and Gibson, Q.H.
Some observations on the kinetics of the reactions with gases of natural and reconstituted haemoglobins.
Biochem. J. 76: 534 (1960).

Antonini, E., Wyman, J., Zito, R., Rossi-Fanelli, A., and Caputo, A.
Studies on carboxypeptidase digests of human hemoglobin.
J. Biol. Chem. 236 PC: 60 (1961).

AOAC
Methods of Analysis AOAC.
Published by The Association of Official Agricultural Chemist, Washington, D.C. (1965).

Atassi, M.Z.
Significance of the amino acid composition of proteins. 1. Compositions of hemoglobins and myoglobins in relation to their structure, function and evolution.
J. Theoret. Biol. 11: 227 (1966).

Atassi, M.Z. and Saplin, B.J.
Studies on myoglobin from the finback whale (Balaenoptera physalus). Preparation, physicochemical and immunochemical characterization, differentiation from sperm-whale myoglobin, amino acid composition and end-terminal analyses.
Biochem. J. 98: 82 (1966).

Banerjee, R.
Etude thermodynamique de l'association Heme-Globine. II. Méthémoglobine.
Biochim. Biophys. Acta 64: 385 (1962).

Bertin-Sans, H. and de Moitessier, I.
Compt. Rend. Acad. Sci. 121: 59 (1893) cited from Rossi-Fanelli et al. (1964).

Bencze, W.L. and Schmid, K.
Determination of Tyrosine and Tryptophan in Proteins.
Anal. Chem. 29: 1193 (1957).

Boardman, N.K. and Adair, G.S.
Isolation of two myoglobins from horse heart extract and the determination of the molecular weight of the main component.
Nature 177: 1078 (1956).

Bodo, G., Dintzis, H.M., Kendrew, J.C., and Wyckoff, H.W.
The crystal structure of myoglobin. V.A. low-resolution three-dimensional Fourier synthesis of sperm-whale myoglobin crystals.
Proc. Royl. Soc. A253: 70 (1959).

Böesken, J.
The use of boric acid for the determination of the configuration of carbohydrates.
Advan. in Carbohydrate Chemistry 4: 189 (1949).

Bowen, W.J.
Notes on myoglobin preparation and iron content.
J. Biol. Chem. 176: 747 (1948).

Brown, W.D., Martinez, M., Johnstone, M., and Olcott, H.S.
Comparative biochemistry of myoglobins.
J. Biol. Chem. 237: 81 (1962).

Cann, J.R.
Kinetics of the reversible reaction of sperm whale myoglobin with zinc.
Biochemistry 3: 714 (1964).

Cann, J.R.
Contribution of aromatic residue interactions to the stability of myoglobin.
Biochemistry 4: 2368 (1965).

Cann, J.R.
Contribution of aromatic residue interactions to the stability of myoglobin. II. Enhancement by aromatic compounds of the rate of urea denaturation.
Biochemistry 6: 3427 (1967a).

Cann, J.R.
Contribution of aromatic residue interactions to the stability of myoglobin. III. Molecular complexes of aromatic compounds with hemin, hematoporphyrin and myoglobin.
Biochemistry 6: 3435 (1967b).

Coryell, C.D. and Pauling, L.
A structural interpretation of the acidity of groups associated with the hemes of hemoglobin and hemoglobin derivatives.
J. Biol. Chem. 132: 769 (1940).

Cullis, A.F., Muirhead, H., Perutz, F.M., Rossman, M.G., and North, A.C.T.
Structure of hemoglobin. IX. A three-dimensional Fourier synthesis at 5.5 Å resolution: description of the structure.
Proc. Roy. Soc. A265: 161 (1962).

Delory, G.E.
The preparation and analysis for iron of haemin and haemoglobin.
The Analyst 68: 5 (1943).

Demmitt, T.F.
Automated procedure for H_3BO_3 analysis in water.
Technicon Symposia. 1965 Automation in analytical chemistry, p. 204.

Drabkin, D.L.
Unaltered globin, and crystalline, synthetic (reconstituted) myoglobin.
J. Biol. Chem. 158: 721 (1945).

Drabkin, D.L.
The distribution of the chromoproteins, hemoglobin, myoglobin and cytochrome c in tissues of different species, and the relationship of the total content of each chromoprotein to body mass.
J. Biol. Chem. 182: 317 (1950).

Edmundson, A.B.
Amino-acid sequence of sperm whale myoglobin.
Nature 205: 883 (1965).

Edmundson, A.B. and Hirs, C.H.W.
Amino-acid sequence of sperm whale myoglobin.
Nature 190: 663 (1961).

Edmundson, A.B. and Hirs, C.H.W.
On the structure of sperm whale myoglobin. I. The amino acid composition and terminal groups of the chromatographically purified protein.
J. Mol. Biol. 5: 663 (1962a).

Edmundson, A.B. and Hirs, C.H.W.
On the structure of sperm whale myoglobin. II. The tryptic hydrolysis of the denatured protein.
J. Mol. Biol. 5: 683 (1962b).

Edmundson, A.B. and Hirs, C.H.W.
On the structure of sperm whale myoglobin. III. Amino acid sequences of the smaller tryptic peptides containing aromatic residues.
J. Mol. Biol. 5: 706 (1962c).

Elson, L.A. and Morgan, W.T.J.
CCXLVIII. A colorimetric method for the determination of glucosamine and chondrosamine.
Biochem. J. 27: 1824 (1933).

Fairbairn, N.J.
A modified anthrone reagent.
Chem. Ind. 72: 86 (1953).

Fraenkel-Conrat, H.
A technique for stepwise degradation of proteins from the amino-end.
J. Am. Chem. Soc. 76: 3606 (1954).

Gibbons, R.A.
Physico-chemical methods for the determination of the purity, molecular size and shape of glycoproteins.
In "Glycoproteins", P. 60, edited by Gottschalk, A.
Elsevier Publishing Company (1966).

Gibson, Q.H. and Antonini, E.
Rates of reaction of native human globin with some hemes.
J. Biol. Chem. 238: 1384 (1963).

Ginger, I.D., Wilson, G.D. and Schweigert, B.S.
Biochemistry of myoglobin. Quantitative determination in beef and pork muscle.
J. Agri. and Food Chem. 2: 1037 (1954).

Gunther
Virchow's Arch. 230: 146 (1921).
cited from Milikan, G.A. (1939).

Hapner, K.D., Bradshaw, R.A., Hartzell, C.R. and Gurd, F.R.N.
Comparison of myoglobins from harbor seal, porpoise, and sperm whale. I. Preparation and characterization.
J. Biol. Chem. 243: 683 (1968).

Hardman, K.D., Eylar, E.H., Ray, D.K., Banaszak, L.J. and Gurd, F.R.N.
Isolation of sperm whale myoglobin by low temperature fractionation with ethanol and metalic ions.
J. Biol. Chem. 241: 432 (1966).

Hill, R. and Holden, H.F.
CLXVI. The preparation and some properties of the globin of oxyhaemoglobin.
Biochem. J. 20: 1326 (1926).

Hirs, C.H.W. and Olcott, H.S.
Amino acid composition of the myoglobin of yellowfin tuna.
Biochim. Biophys. Acta 82: 178 (1964).

Inada, Y. and Shibata, K.
The soret band of monomeric hematin and its changes on polymerization.
Biochem. Biophys. Res. Comm. 9: 323 (1962).

Jacin, H. and Mishkin, A.R.
Separation of carbohydrates on borate-impregnated silica gel G plates.
J. of Chromatography 18: 170 (1965).

Karadzova, M., Nedkov, P.T., Atanassov, B. and Keil, B.
Coll. Czech. Chem. Comm. 29: 551 (1964).
cited from Atassi and Saplin (1966).

Kendrew, J.C., Parrish, R.G., Marrack, J.R. and Orlans, E.S.
The species specificity of myoglobin.
Nature 174: 946 (1954).

Kendrew, J.C., Bodo, G., Dintzis, H.M., Parrish, R.G. Wyckoff, H. and Phillips, D.C.
A three-dimensional model of the myoglobin molecule obtained by X-ray analysis.
Nature 181: 662 (1958).

Kendrew, J.C., Dickerson, B.E., Strandberg, B.E., Hart, R.G., Davies, D.R., Phillips, D.C. and Shore, V.C.
Structure of myoglobin. A three-dimensional fourier synthesis at 2 Å resolution.
Nature 185: 422 (1960).

Kendrew, J.C., Watson, H.C., Strandberg, B.E., Dickerson, R.E., Phillips, D.C. and Shore, V.C.
The amino acid sequence of sperm whale myoglobin. A partial determination by X-ray methods and its correlation with chemical data.
Nature 190: 666 (1961).

Keyser, J.W.
Staining of serum glycoproteins after electrophoretic separation in acrylamide gels.
Anal. Biochem. 9: 249 (1964).

Konosu, S., Hashimoto, K. and Matsuura, F.
Bull. Jap. Soc. Sci. Fish. 24: 513 (1958).
cited from Atassi and Saplin (1966).

Lein, A. and Pauling, L.
The combining power of myoglobin for alkyl isocyanides and the structure of the myoglobin molecule.
Proc. Natl. Acad. Sci. U.S. 42: 51 (1956).

Lemberg, R. and Legge, J.W.
Hematin compounds and bile pigments. Their constitution, metabolism, and function.
Interscience Publishers, Inc., New-York (1949).

Lewis, U.J.
Acid cleavage of heme proteins.
J. Biol. Chem. 206: 109 (1954).

Lewis, U.J. and Schweigert, B.S.
Biochemistry of myoglobin. III. Homogeneity studies with crystalline beef myoglobin.
J. Biol. Chem. 214: 647 (1955).

Luginbuhl, W.H.
A method of crystallization of human myoglobin.
Proc. Soc. Exptl. Biol. Med. 105: 504 (1960).

Lumry, R.
Biophysics (Japan) 138 (1961).
cited from Rossi-Fanelli et al. (1964).

Mac Munn.
Proc. Roy. Soc. 31: 206 (1881).
cited from Millikan (1939).

McKinley, W.P. and Read, S.I.
Use of starch gel electrophoresis to illustrate inhibition of rabbit liver esterases by organophosphorus pesticides.
J.A.O.A.C. 44: 726 (1961).

Millikan, G.A.
Muscle hemoglobin.
Physiol. Review 19: 503 (1939).

Morgan, V.E.
Studies on myoglobin. I. The solubility of myoglobin in concentrated ammonium sulfate solutions.
J. Biol. Chem. 112: 557 (1936).

Owen, J.A., Silberman, H.J. and Got, C.
Detection of Haemoglobin, Haemoglobin-Haptoglobin Complexes and other Substances with Peroxidase Activity after Zone Electrophoresis.
Nature 182: 1373 (1958).

Pastuska, G.
Untersuchungen über die qualitative und quantitative Bestimmung der Zucker mit Hilfe der Kieselgelschicht-Chromatographie. II. Mitteilung.
Z. Analyt. Chem. 179: 427 (1961).

Perkoff, G.T., Hill, R.L., Brown, D.M., and Tyler, F.H.
The characterization of adult human myoglobin.
J. Biol. Chem. 237: 2820 (1962).

Perutz, M.F., Rossmann, M.G., Cullis, A.F., Muirhead, H., Will, G. and North, A.C.T.
Structure of haemoglobin. A three-dimensional fourier synthesis at 5.5 Å resolution obtained by X-ray analysis.
Nature 185: 416 (1960).

Perutz, M.F., Kendrew, J.C. and Watson, H.C.
Structure and function of haemoglobin. II. Some relations between polypeptide chain configuration and amino acid sequence.
J. Mol. Biol. 13: 669 (1965).

Peterson, E.A. and Sober, H.A.
Chromatography of proteins. I. Cellulose ion-exchange adsorbents.
J. Am. Chem. Soc. 78: 751 (1956a).

Peterson, E.A. and Sober, H.A.
Chromatography of proteins. II. Fractionation of serum protein on anion-exchange cellulose.
J. Am. Chem. Soc. 78: 756 (1956b).

Polson, A.
Untersuchungen über die Diffusionskonstanten der Proteine.
Kolloid Zeitschrift 87: 149 (1939).

Poulik, M.D.
Starch gel electrophoresis in a discontinuous system of buffers.
Nature 180: 1477 (1957).

Roche, J., Derrien, Y. et Viel, H.
Recherches sur la composition et la spécificité des myoglobins de divers mammifères.
Trav. members soc. chim. biol. 24: 1016 (1942).

Rossi, A.
Sulla costituzione chimica della mioglobina.
Arch. Sc. Biol. 26: 244 (1940).

Rossi, A. and Travia, L.
Specificita è composizione chimica di mioglobina di specie diverse.
Boll. Soc. Ital. Biol. Sper. 16: 768 (1941).

Rossi-Fanelli, A.
Crystalline human myoglobin: some physicochemical properties and chemical composition.
Science 108: 15 (1948).

Rossi-Fanelli, A. and Antonini, E.
Reversible splitting of human myoglobin. Physico-chemical properties and oxygen equilibrium of reconstituted proto- and deutero-myoglobin.
Arch. Biochem. Biophys. 72: 243 (1957).

Rossi-Fanelli, A. and Antonini, E.
The oxygen equilibrium of reconstituted hemoglobins. I. Reconstituted human proto hemoglobin.
Arch. Biochem. Biophys. 80: 299 (1959a).

Rossi-Fanelli, A. and Antonini, E.
The oxygen equilibrium of reconstituted hemoglobins. II. Reconstituted human deuterohemoglobin.
Arch. Biochem. Biophys. 80: 308 (1959b).

Rossi-Fanelli, A. and Antonini, E.
Dissociation of hematin from hemoproteins at neutral pH.
J. Biol. Chem. 235: PC4 (1960).

Rossi-Fanelli, A., Antonini, E. and Caputo, A.
Pure native globin from human hemoglobin: preparation and some physico-chemical properties.
Biochim. Biophys. Acta 28: 221 (1958a).

Rossi-Fanelli, A., Antonini, E. and Caputo, A.
Studies on the structure of hemoglobin. I. Physicochemical properties of human globin.
Biochim. Biophys. Acta 30: 608 (1958b).

Rossi-Fanelli, A., Antonini, E. and Povoledo, D.
Further study on myoglobin. II. Chemical and biochemical properties of a new type of myoglobin in molluscus.
"Symposium on Protein Structure." p. 144 (1958c).
Edited by Neuberger A. John Wiley and Sons Inc.

Rossi-Fanelli, A., Antonini, E. and Caputo, A.
Hemoglobin and myoglobin.
Adv. Prot. Chem. 19: 73 (1964).

Rumen, N.M.
Isolation of five myoglobins from seal.
Acta Chem. Scand. 13: 1542 (1959).

Rumen, N.M.
Amino acid composition of seal myoglobin I.
Acta Chem. Scand. 14: 1217 (1960).

Rumen, N.M. and Appella, E.
Molecular association behavior of apomyoglobin I from the seal.
Arch. Biochem. Biophys. 97: 128 (1962).

Schachman, H.K.
Ultracentrifugation, diffusion and viscometry.
Methods in Enzymology 4: 32 (1957).

Schmid, K.
Electrophoretic properties and analysis of whale myoglobin.
Nature 163: 481 (1949).

Schönheimer, R.
Über den roten Farbstoff der Hertz und Skelett muskulatur.
Z. Physiol. Chem. 180: 144 (1929).

Scopes, R.K.
Isolation and properties of a basic protein from skeletal-muscle sarcoplasm.
Biochem. J. 98: 193 (1966).

Scouloudi, H.
The crystal structure of myoglobin. VI. Seal myoglobin.
Proc. Royl Soc. A258: 181 (1960).

Scouloudi, H. and Prothero, J.W.
The nature and configuration of the mercuri-iodide ion in the seal myoglobin derivative.
J. Mol. Biol. 12: 17 (1965).

Shack, J. and Clark, W.M.
Metalloporphyrins. VI. Cycles of changes in systems containing heme.
J. Biol. Chem. 171: 143 (1947).

Signor, A., Previero, A. and Terbojevich, M.
Determination of the N-terminal amino-acid in polypeptides and proteins.
Nature 205: 596 (1965).

Smith, M.H. and Gibson, Q.H.
The preparation and some properties of myoglobin containing meso- and deutero-haem.
Biochem. J. 73: 101 (1959).

Smith, E.L. and Stockell, A.
Amino acid composition of crystalline carboxypeptidase.
J. Biol. Chem. 207: 501 (1954).

Smith, E.L., Stockell, A. and Kimmel, J.R.
Crystalline papain. III. Amino acid composition.
J. Biol. Chem. 207: 551 (1954).

Smithies, O.
Zone electrophoresis in starch-gels: Group variations in the serum proteins of normal human adults.
Biochem. J. 61: 629 (1955).

Smithies, O.
An improved procedure for starch-gel electrophoresis: Further variations in the serum proteins of normal individuals.
Biochem. J. 71: 585 (1959a).

Smithies, O.
Zone electrophoresis in starch-gels and its application to studies of serum proteins.
Advan. Prot. Chem. 14: 141 (1959b).

Spackman, D.H., Stein, W.H. and Moore, S.
Automatic recording apparatus for use in the chromatography of amino acids.
Anal. Chem. 30: 1190 (1958).

St. George, R.C.C. and Pauling, L.
The combining power of hemoglobin for alkyl isocyanides, and the nature of heme-heme interactions in hemoglobin.
Science 114: 629 (1951).

Stahl, E.
Thin-layer chromatography.
Edited by Stahl, E. Academic Press Inc. (1965).

Stryer, L., Kendrew, J.C. and Watson, H.C.
The mode of attachment of the azide ion to sperm whale metmyoglobin.
J. Mol. Biol. 8: 96 (1964).

Svedberg, T.
The ultra-centrifuge and the study of high-molecular compounds.
Nature 139: 1051 (1937).

Theorell, H.
Kristallinisches Myoglobin. I. Kristallisiern und Reinigung des Myoglobins, sowie vorlänfige Mitteilung über sein Molekulärgewicht.
Biochem. Zeitschr. 252: 1 (1932).

Theorell, H.
Kristallinesches Myoglobin. II. Metteilung Sedimentationskonstante und Molekulärgewicht des Myoglobins.
Biochem. Zeitschr. 268: 46 (1934a).

Theorell, H.
Kristallinesches Myoglobin. III. Die absolute Lichtabsorption von Oxy-, Carboxy-, Meta- und reduzierten Myoglobin.
Biochem. Zeitschr. 268: 55 (1934b).

Theorell, H.
Kristallinesches Myoglobin. IV. Myoglobin im Gleichgewicht mit Sauerstoff und Kohlenoxyd.
Biochem. Zeitschr. 268: 64 (1934c).

Theorell, H.
Kristallinesches Myoglobin. V. Die Sauerstoff-bindungskurve des Myoglobin.
Biochem. Zeitschr. 268: 73 (1934d).

Theorell, H. and Akeson, A.
Reversible splitting of a homogeneous horse myoglobin.
Ann. Acad. Sci. Fennicae Ser. AII 60: 303 (1955).
cited from Cehm. Abstr. 49: 11739 h (1955).

Theorell, H. and de Duve, C.
Crystalline human myoglobin from heart muscle and urine.
Arch. Biochem. 12: 113 (1947).

Timmer, R., van der Helen, H.J. and Huisman, T.H.J.
Myoglobin from sheep homozygous for different haemoglobin types.
Nature 180: 240 (1957).

Trevelyan, W.E., Procter, D.P. and Harrison, J.
Detection of sugars on paper chromatograms.
Nature 166: 444 (1950).

Wang, J.H., Nakahara, A. and Fleischer, E.B.
Hemoglobin studies. 1. The combination of carbon monoxide with hemoglobin and related model compounds.
J. Am. Chem. Soc. 80: 1109 (1958).

Warren, L.
The thiobarbituric acid assay of sialic acids.
J. Biol. Chem. 234: 1971 (1959).

Wyman, J.
Heme proteins.
Adv. Prot. Chem. 4: 407 (1948).