ABSTRACT

ISOLATION AND CHARACTERIZATION OF MULTIPLE

BOVINE MYOGLOBINS

by John Robert Quinn

The heme-containing, pigmented protein myoglobin is chiefly responsible for the color of lean meat. The basic cause of meat color change can best be elucidated from studies on the pure pigment. Myoglobin of crystalline purity has been extensively characterized. Recently, however, the heterogeneity of crystalline bovine myoglobin and the multiple occurrence of myoglobin in several other species has been demonstrated.

This study was undertaken to separate and isolate the multiple myoglobins of bovine muscle. Also, characterization of the multiple myoglobins was initiated with the aim of determining specific differences between them, and by so doing to evaluate the significance of their multiple occurrence.

The presence of at least 3, and probably 4, distinct myoglobins was detected by starch gel electrophoresis. The 3 major myoglobins, labelled Mb I_A, Mb I_B and Mb II and comprising roughly 10-15, 2-5 and 80-88 percent, respectively, of the total myoglobin, were separated and isolated in fairly homogeneous form by cellulose ion exchange chromatography. Various chromatographic techniques were tested in both DEAE cellulose and carboxymethyl cellulose columns. The ultimately-adopted chromatographic procedure involved a stepwise development technique utilizing both tris and phosphate buffers on DEAE cellulose columns.

Characterization studies on the 3 myoglobins revealed them to be similar in light absorption minima and maxima wavelength positions for

both their met- and carboxy-derivatives, in heme contents and in autoxidation rates. The autoxidation rates for all 3 myoglobins at high temperatures (29-33°C) were found to be higher than literature values. The rates constants were also higher than values found in this laboratory for less homogeneous samples.

The 3 myoglobins were found to be dissimilar in spectral absorptivity values for both their met- and carboxy-derivatives, in ease of heme cleavage by acid, and in degree of inhibiting the reduction of cytochrome c by xanthine oxidase. The dissimilarities among the 3 myoglobins suggested differences in their heme-globin linkages.

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INTRODUCTION

Color is of fundamental importance in the marketing of meat. Under conditions of commercial retailing, discoloration is probably the major factor in determining the shelf life of prepackaged meat cuts. The color of lean meat is largely dependent upon the quantity and the chemistry of the pigmented heme-protein, myoglobin. When the iron of the heme group is in the divalent state, myoglobin can react with atmospheric oxygen to form either the bright red oxygenated pigment of normal meat, oxymyoglobin, or the brown oxidized ferric compound, metmyoglobin. It is the formation of the brown color caused by autoxidation of myoglobin that presents major concern to the meat industry.

The basic cause, or chemistry, of color change in meat can best be elucidated from studies on the pure pigment. Myoglobin has been extensively characterized in systems of defined purity with the degree of purity usually being defined by its crystallization.

Crystalline myoglobin preparations from several animal species have been reported to contain multiple, electrophoretically-distinct myoglobins. Lewis and Schweigert (1955) found that crystalline beef muscle myoglobin was electrophoretically heterogeneous, but did not recognize the heterogeneity as being due to the multiple occurrence of the protein. However, a preliminary attempt in this laboratory to purify bovine myoglobin by chromatography indicated its multiple occurrence.

The significance of the multiple occurrence of myoglobin to both meat color and animal physiology can be evaluated only by investigating

the properties of the individual myoglobins. Thus, the aims of this study were to separate, isolate and characterize the myoglobins of beef muscle.

REVIEW OF LITERATURE

Concentration of Myoglobin in Beef Muscle

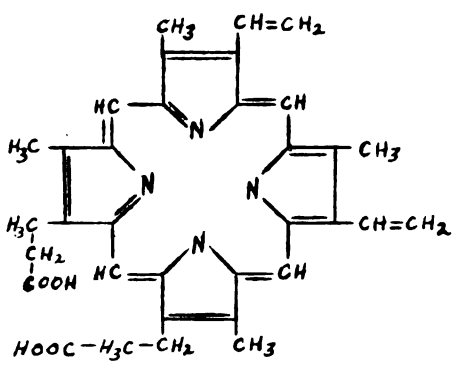
Estimations on the myoglobin content of muscle have involved water extraction of the total pigment, including residual hemoglobin, and spectrophotometric measurement of a myoglobin derivative. Ginger et al. (1954) determined myoglobin as the cyanide met-derivative after salting the hemoglobin from solution. They reported average values of 3.7 and 2.1 mg. per g. of fresh tissue for beef skeletal and heart muscle, respectively. Fleming et al. (1960) found that measurement of the carbon monoxide derivative of myoglobin provided the most satisfactory estimation of myoglobin in muscle extracts. The average myoglobin concentration in mg. per g. was reported to be 3.27 for beef skeletal muscle. Shank, ribeye, sirloin tip and heart muscle had myoglobin contents of 3.22, 3.51, 2.97 and 2.40, respectively. Lawrie (1960) also converted the extracted pigment to its carboxy-derivative, and reported the myoglobin content of beef longissimus dorsi muscle to increase with the age of the animal. From 0.05 percent of the wet tissue at birth, the myoglobin content increased to 0.40 percent at 20 to 24 months of age and to 0.6 percent at 5 to 6 years.

General Chemistry of Myoglobin

Structural Properties

Myoglobin is a conjugated protein composed of a basic histone, globin, and a heme prosthetic group (West and Todd, 1957). In a compre-

hensive review of the early literature on heme compounds, Lemberg and Legge (1949) describe the heme group as composed of 4 pyrrole nuclei joined together into a planar ring by methene bridges. An iron atom, located in the center of the ring, is coordinately bonded to each of the 4 pyrrole nitrogens. Lemberg and Legge (1949) pointed out that the chemical formula of the tetrapyrrole, or porphyrin, is characterized by a large number of alternating double bonds and that, consequently, the structure is very highly resonant with a resultant deep color. The many different porphyrins existing in nature vary from one another in their side chains, which substitute the 8 hydrogen atoms in the B-positions of the pyrrole nuclei. Protoporphyrin IX, as taken from Lemberg and Legge (1949), is reproduced below.



Protophorphyrin IX is the porphyrin of myoglobin, hemoglobin, catalase, and peroxidase. It contains 2 vinyl, 4 methyl, and 2 propionic acid side chains.

The iron-porphyrin, or heme, complex in native myoglobin is held near the surface of the globin molecule, with one side apparently exposed for the attachment of oxygen or other ligands (Kendrew, 1959). Kendrew

et al. (1961) implicated 3 imidazoles in the binding of the heme to the globin. One histidine is coordinated directly to the iron, a probable histidine is linked indirectly to iron through a H_2O molecule, and another histidine is apparently hydrogen-bonded to a heme propionic acid side chain. The whole inner part of the heme group is surrounded by non-polar protein side chains.

In structure and properties, the sarcoplasmic protein myoglobin resembles the more thoroughly studied blood protein hemoglobin. Specific similarities and differences between the 2 proteins are discussed throughout this review.

According to Lemberg and Legge, (1949) both myoglobin and hemoglobin are water-soluble. Both have the same equivalent weight based on the number of oxygen-combining, or heme, groups. The iron content for each of the proteins has been determined by Drabkin (1949) to be 0.34 percent. However, myoglobin has a molecular weight of approximately 17,000 and is composed of a single, randomly coiled polypeptide chain and a single heme group, whereas hemoglobin has an approximate molecular weight of 67,000 and is composed of 4 polypeptide chains, each associated with a heme group (Kendrew, 1958). Hemoglobin can be split into 4 sub-units by various treatments. The 4 polypeptide chains are of roughly equal size and are of 2 types, which are denoted α and β (Perutz et al., 1960).

Kendrew et al. (1961) has correlated x-ray data with the preliminary chemical results of Edmunson and Hirs (1961) to tentatively identify the amino acid sequence of sperm whale myoglobin. A total of 152 or 153 amino acid residues form the single polypeptide chain. Kendrew et al.

(1960) found that about 65 to 72 percent of the molecule consists of right-handed α -helices, made up of about 8 segments. Each segment contains 7 to 20 residues. The helical structure is disrupted, usually for 2 or 3 residues, when the chain turns a corner.

The similarities in primary structure of myoglobins from different species have been discussed by Kendrew (1959). The amino acid compositions of the different myoglobins are broadly similar, but differ in detail. In some cases, myoglobins from 2 different species crystallize in forms so similar that their x-ray patterns are virtually identical. Sperm whale and porpoise myoglobins, for example, give x-ray patterns which can hardly be distinguished. Horse and seal myoglobins, likewise, form a crystalline pair. Kendrew (1959) points out that such near identity of diffraction patterns implies that the molecules must have virtually the same secondary and tertiary structures.

Perutz et al. (1960) have shown that the tertiary structures of the α and β chains of horse hemoglobin closely resemble those of sperm whale myoglobin. Watson and Kendrew (1961), however, observed a correspondence (identical residues in corresponding positions) of only 28 percent between sperm whale myoglobin and either the α or β chains of human hemoglobin. Tristram (1949) found horse hemoglobin and horse myoglobin to be completely dissimilar in amino acid composition. Hemoglobin contains both cysteine and cystine residues, whereas myoglobin contains neither of these amino acids. Sixty-two percent of the basic groups in myoglobin are due to lysine and 31 percent to histidine, whereas lysine and histidine each contribute 40 percent of the basic groups of hemoglobin.

Brown et al. (1962), in a comparative study of myoglobins obtained from various sources, reported that, in contrast to avian and mammalian myoglobins, fish myoglobins contain free sulfhydryl groups.

The electrophoretic and chromatographic behavior of myoglobins from several different species is discussed in a later section of this review.

Lemberg and Legge (1949) describe all porphyrin compounds as absorbing light energy in the 400-450 mu (Soret band) region of the electromagnetic spectrum. The positions of absorption maxima at other wavelengths are, in general, characteristic for each individual porphyrin, that is, they are dependent upon the porphyrin side chains. When a porphyrin combines with iron, or any metal ion, its spectrum changes in the visible region. When a heme combines with a protein imidazole group and a H_2O molecule, as is the case for myoglobin and hemoglobin, the intensity and position of the absorption bands are affected only slightly (Williams, 1956). Brill and Williams (1961) correlated the absorption spectra of several heme protein derivatives with magnetic susceptibility data. They developed a diagnostic procedure by which the type of group bound to the heme could be determined by spectroscopy alone.

The properties of heme and its derivatives are related to the electronic state of the iron atom. As summarized from Lemberg and Legge (1949), the iron ion has 23 (ferric ion) or 24 (ferrous ion) extranuclear electrons, of which 18 form an argon core. The remaining 5 or 6 electrons are in the outermost (M) shell and are distributed as shown:

	Orbitals			Number of unpaired electrons
	3d	4s	4p	
ferrous ionic	⚊⚊ ⚊ ⚊ ⚊ ⚊	⚊	⚊ ⚊ ⚊	4
ferrous covalent	⚊⚊ ⚊⚊ ⚊⚊ ⚊ ⚊	⚊	⚊ ⚊ ⚊	0
ferric ionic	⚊ ⚊ ⚊ ⚊ ⚊	⚊	⚊ ⚊ ⚊	5
ferric covalent	⚊⚊ ⚊⚊ ⚊ ⚊ ⚊	⚊	⚊ ⚊ ⚊	1

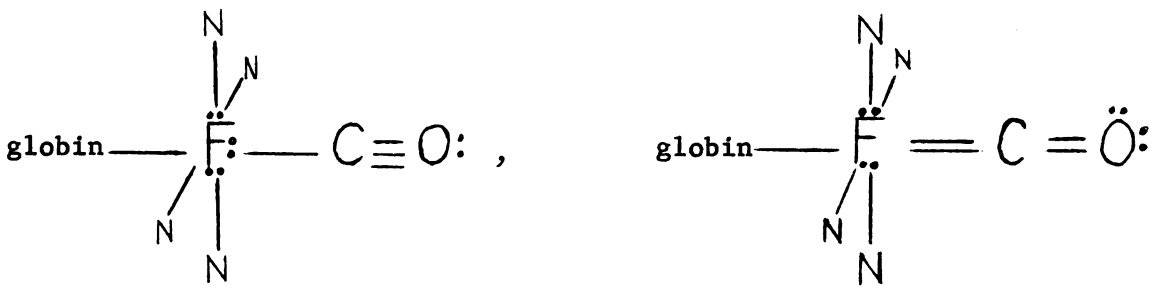
Both ionic (high-spin) and covalent (low-spin) bonding of the iron can occur. In ionic bonding, the 3d orbitals are unaffected, and consequently, the compound is associated with a large number of unpaired electrons. The iron is held in combination by the electrostatic attraction between its own ionic charge and the charge on the surrounding ions of the complex.

According to Lemberg and Legge (1949), the pairing of the single electrons in the 3d orbitals to form 6 available orbitals allows the acceptance of 12 electrons from other atoms, with resultant formation of electron-pair or covalent bonds. Since the 3d, 4s, and 4p orbitals have but slightly differing stabilities, hybridization takes place resulting in the production of 6 symmetrically disposed, energetically equivalent bond orbitals. The bond orbitals are directed towards the corners of a regular octahedron.

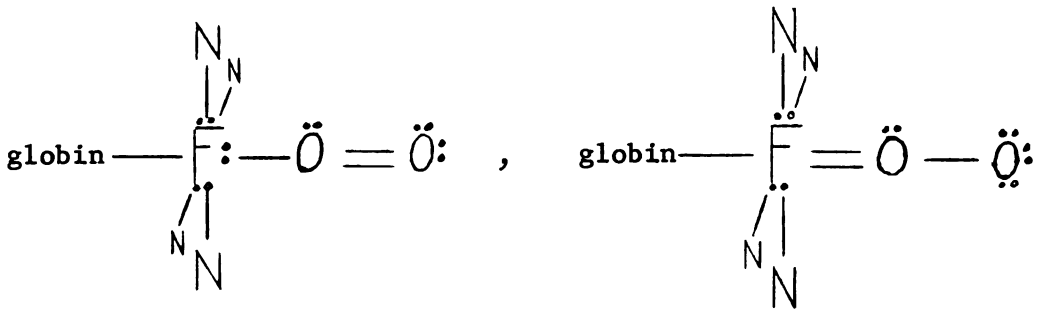
When electrons are paired in an orbital, their spins are opposed. According to Fruton and Simmonds (1958), if an electron is unpaired its unopposed spin confers a permanent magnetic moment on the molecule, and the molecule is repelled by an external magnetic field, that is, it is paramagnetic. When all electrons in a compound are paired, the compound is described as being diamagnetic.

The magnetic susceptibilities of both hemoglobin and myoglobin, although differing slightly in numerical value per heme group (Brill and Williams, 1961), lead to the same structural postulations. Hence, for purposes of this review both proteins are included under the general term, the heme proteins or pigments.

Pauling and Coryell (1936a) studied the magnetic properties of hemochromagen (reduced heme attached to denatured globin) and found it diamagnetic. They concluded that the hemochromagen spectrum, characterized by 2 sharp bands, α at about 560 mu and β at about 520 mu, correlated with a structure in which the iron atom is attached by essentially covalent bonds, not only to the 4 porphyrin nitrogen atoms, but also to 2 other atoms. The same authors (1936b) found the oxy- and carboxy- heme protein derivatives to be diamagnetic, and the reduced pigments to contain 4 unpaired electrons per heme. The carboxy- system was depicted as resonating between 2 structures as shown:



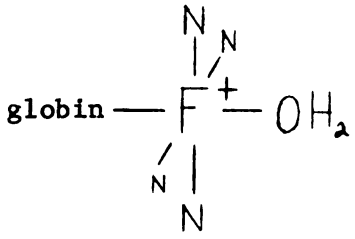
The dashes represent shared electron pairs and the dots unshared electrons. Similarily, the oxygenated heme proteins were represented as resonating between the 2 structures:



Pauling (1948) later explained the power of specific combination with oxygen and carbon monoxide possessed by the reduced heme proteins by the postulate of the approximate electrical neutrality of all atoms in stable compounds. The iron atom is made approximately neutral by the bonds to the nitrogens of the porphyrin, and accordingly, only a molecule that can form covalent bonds with the iron atom without transferring a large amount of electrical charge to this atom would be expected to combine with the reduced heme proteins. Oxygen, carbon monoxide and cyanide ion have the ability to share electrons, forming a double bond with the iron, and, hence, can combine. Electron donor groups, such as water molecules, chloride ions, hydroxide ions, etc., cannot combine. Pauling and Coryell (1936b) showed that there are 4 unpaired electrons per heme in the reduced pigments and that, consequently, the iron atom in these compounds is not attached to the 4 porphyrin nitrogen atoms and the globin molecule by covalent bonds, but that the bonds are essentially of an ionic type.

The absorption spectra of the oxy- and carboxy- heme pigments show 2 sharp bands in the yellow-green region. The absorption maxima for oxymyoglobin and carboxymyoglobin occur at wavelengths 582 and 544 mu and at 577 and 540 mu, respectively (Bowen, 1949). Reduced myoglobin exhibits one broad absorption band at 555 mu (Bowen, 1949).

Coryell et al. (1937) found 5 unpaired electrons per heme in the oxidized, or met-, heme proteins. They concluded that the bonds to the iron in these compounds were of an ionic character. The met- pigments may be written as having the formal structure:



The spectrum of metmyoglobin is characterized by absorption maxima at 630 mu and at 500 mu (Bowen, 1949).

The met- pigment carries a positive charge and as the pH becomes greater than 7, the sixth bond position of the iron becomes occupied by a hydroxyl ion and the color changes from brown to bright red. Coryell et al. (1937) demonstrated that a change in magnetic susceptibility occurs to indicate the presence of 3 unpaired electrons per heme and, consequently, a bonding 2/3 covalent and 1/3 ionic. The absorption band at 630 mu diminishes in intensity and shifts to 600 mu, while the weaker bands in the remainder of the spectrum (580 and 540 mu) become more pronounced (Lemberg and Legge, 1949). The 2 forms of met- pigment are in equilibrium with each other as shown by the following equation:

$$\text{Met } Fe^{+} - OH_2 \rightleftharpoons \text{Met Fe} - OH + H^{+}$$

where Met $Fe^{+} - OH_2$ represents acid met- pigment and Met Fe - OH represents alkaline met- pigment. The pK of this reaction was found to be 8.15 for methemoglobin (Coryell et al., 1937) and 8.9 for horse metmyoglobin (George and Hanania, 1952).

When cyanide ion is added to solutions of the met- pigments, a bright red color develops and the spectra is characterized by an absorption maximum at 540 mu (Bowen, 1949). Coryell et al. (1937) demonstrated the presence of one unpaired electron per heme group and, consequently, iron covalent bonding.

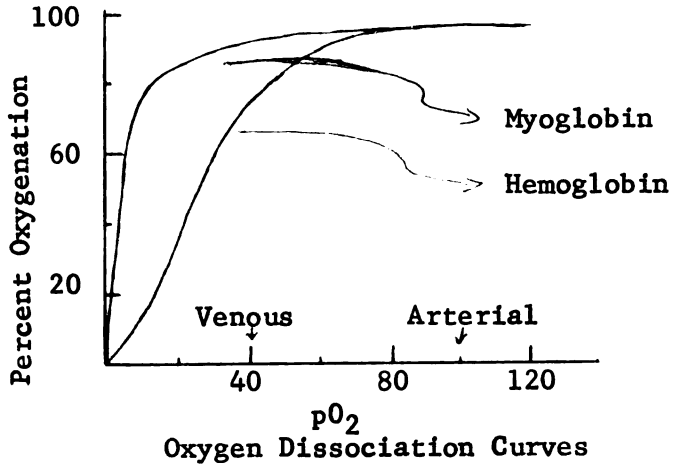
Functional Properties and Physiological Role

According to Watts (1954), the outstanding property of both hemoglobin and myoglobin lies in their ability to combine reversibly with molecular oxygen. This ability depends upon their specific heme- protein linkage. Denaturation of the globin destroys the oxygen combining property and greatly increases the susceptibility of the heme to oxidative degradation.

The oxygenation reaction of myoglobin may be written:

$$\text{Myoglobin (Mb)} + O_2 \rightleftharpoons \text{oxymyoglobin } (MbO_2)$$

The equilibrium constant of the reaction is $Ke = \frac{(MbO_2)}{(Mb)\ (O_2)}$, or since the concentration of O_2 is proportional to the partial pressure of the gas, $K = \frac{(MbO_2)}{(Mb)\ pO_2}$. This final relationship is usually termed Hüfners' equation (Lemberg and Legge, 1949). If the percent saturation $Y = \frac{(MbO_2)}{(MbO_2 + Mb)}$ is plotted against pO_2, the curve takes the form of a rectangular hyperbola. Oxygen dissociation curves of myoglobin and hemoglobin are taken from Fruton and Simmonds (1958) and reproduced below.



Oxygen Dissociation Curves

The dissociation curve of hemoglobin is sigmoid in shape and is described by the more complicated Hill equation (Lemberg and Legge, 1949). Increased p CO_2 shifts the dissociation curve of hemoglobin to the right (the Bohr effect), presumably by increasing the acidity of the solution, but does not affect myoglobin oxygenation (White et al., 1959).

As is noted from the dissociation curves, oxymyoglobin is less dissociated than hemoglobin at the venous pressure of oxygen. The higher affinity of myoglobin for oxygen is of physiological significance, because, according to Fruton and Simmonds (1958), it facilitates the transfer of oxygen from oxyhemoglobin to the sites of oxidation in the muscle cell.

Milliken (1937) stated that the function of myoglobin as an oxygen store is of greater physiological importance than its function in oxygen transfer. The storage function of myoglobin was described as that of providing a supply of oxygen when the need is greatest, as well as in smoothing out the fluctuations in oxygen content during intermittent action. Lawrie (1953) demonstrated that variations in the activities of the cytochrome system were directly paralleled by corresponding variations in the percentage of myoglobin in the whole tissue, and that

this parallelism extended over muscles differing in age, type, and species. Lemberg and Legge (1949) stated that myoglobin is 94 percent saturated at the oxygen tension of venous blood and about 50 percent of this oxygen could be dissociated at the apparent loading tension of cytochrome oxidase. To function as an oxygen store a pigment must also be able to load and unload its oxygen with sufficient speed. Milliken (1937) measured the rate of dissociation of oxygen from oxymyoglobin during tetanic contraction of the soleus muscle of the cat. It was shown that the reaction rate was of the same order as that of the increase of muscular tension during contraction.

The velocities with which oxygen and carbon monoxide react with myoglobin and hemoglobin are taken from Lemberg and Legge (1949) and reproduced below:

	Velocity constants		
Reaction	myoglobin	hemoglobin	
O_2 association	19,000	4,000	millimoles^{-1} sec^{-1}
O_2 dissociation	37	40	sec^{-1}
CO association	300	130	millimoles^{-1} sec^{-1}
CO dissociation	0.04	0.004	sec^{-1}

With the exception of the dissociation of oxymyoglobin, all the myoglobin reactions are faster than the corresponding hemoglobin reactions. Lemberg and Legge (1949) pointed out that there is reasonably good agreement between the affinity constants of both myoglobin and hemoglobin for the 2 gases and the ratio of their respective velocity constants for the forward and reverse reactions. These authors pointed out that the

agreement is not unexceptional in the case of myoglobin, since the myoglobin dissociation curve may be accurately described by the Hüfner equation, but that it is unexplained for hemoglobin.

According to Ramsey (1949), the percentage of hemoglobin in normal blood that exists as the oxidized, or met-, derivative is very small (0.5 percent or less). The general reducing conditions, which are found in the erythrocyte and which are promoted primarily by the action of oxidative enzyme systems, keep the iron of hemoglobin in the ferrous state. Similarily, reducing conditions in living muscle keep the iron of myoglobin largely in the divalent state. The survival of several oxidative enzyme systems following death and storage of the muscle has been demonstrated by Andrews et al. (1952) and Grant (1955). It has been often observed that freshly-cut meat has a deep purplish-red color characteristic of reduced myoglobin. Upon exposure to the atmosphere, a bright red color characteristic of oxymyoglobin is formed. After prolonged exposure, a brown color typical of metmyoglobin gradually replaces the bright red.

Autoxidation of Myoglobin

The oxidation-reduction potentials of myoglobin and hemoglobin have been thoroughly studied by various authors including Conant (1923), Conant and Fieser (1924), Conant and Scott (1926, 1928), Taylor and Hastings (1939), and Taylor and Morgan (1942). These studies have clearly shown the oxidized-reduced pigment systems to be thermodynamically reversible.

Taylor and Morgan (1942) established the involvement of one electron in the myoglobin redox system (E_0 value of + 0.046 volts at pH 7.0 and 30°C).

Credit for the first clue that ease of oxidation of the red-colored oxy- pigments to their brown met- derivatives might increase under environments of low oxygen tensions should probably be given to Conant and Fieser (1924). They found that whereas hemoglobin in the absence of oxygen could be completely oxidized by one equivalent of ferricyanide, 13.5 equivalents were required at oxygen pressures near one atmosphere.

Further work by Neil and Hastings (1925) and Brooks (1929, 1935) confirmed this belief. They observed that the rate of oxidation of hemoglobin by oxygen reached a maximum at oxygen tensions of 4 and 20 mm. Hg. at temperatures of 0°C and 30°C, respectively, and that under these conditions the hemoglobin molecule was half oxygenated. This observation suggested to Neil and Hastings (1925), and later to Brooks (1931, 1935), that methemoglobin was formed by the oxidation of hemoglobin rather than of oxyhemoglobin. The mechanism proposed by Brooks, and involving an inhibitory effect of oxygen, was later criticized by Lemberg and Legge (1949). Neil and Hastings (1925) and Brooks (1929) found no evidence of methemoglobin formation in the complete absence of oxygen.

Under constant conditions of oxygen pressure, pH, salt concentration, and temperature, Brooks (1931) found the rate of the autoxidation reaction to be first order with respect to unoxidized myoglobin. He also found the rate constant to increase with a decrease of pH or an increase in phosphate concentration.

The autoxidation of solutions of crystallized horse heart myoglobin was studied by George and Stratmann (1952a, 1952b, 1954). In the first paper, the authors reported that under constant conditions of pH, temperature, and phosphate concentration (5.69, 30°C, and 0.6 M, respectively), the autoxidation of myoglobin in air was first order in unoxidized myoglobin and had a mean rate constant value of 0.325 ± 0.015 $hour^{-1}$. Measurement of the oxygen absorbed during autoxidation showed that 2.5 moles of oxygen were consumed per mole of metmyoglobin formed. The absorption of oxygen was also a first order reaction.

In the second paper, the authors reported myoglobin autoxidation to be first order in percentage of unoxidized pigment over a range of oxygen pressures from 0.3 to 760 mm Hg. At 30°C, the rate constant (k) at first increased with increasing oxygen pressures, exhibited a well-defined maximum value at 1 - 1.4 mm partial pressure, and then decreased to a constant value above 30 mm. The determination of the equilibrium constant (Ke) for the reaction:

$$\text{myoglobin} + O_2 \rightleftharpoons \text{oxymyoglobin}$$

under the conditions of the autoxidation experiments gave a value of 0.88 ± 0.12 mm^{-1}. Hence, the partial pressure for half saturation (1 - 1.31 mm) and for the maximum rate of autoxidation were found to be the same. Using the value of Ke, the variation of k with oxygen pressure was shown to fit the equation:

$$k = 2.30 \times Mb \times MbO_2 + 0.30 \times MbO_2,$$

where Mb and MbO_2 represent the fractions of reduced and oxy-myoglobin, respectively.

George and Stratmann (1952b) proposed a free radical mechanism for the autoxidation of myoglobin. The mechanism involves the participation of an auxiliary electron-accepting group on the protein molecule, which, acting as a catalyst in a separate reaction, regenerates the unoxidized myoglobin. The proposed mechanism, however, presumes the formation of hydrogen peroxide during the autoxidation and fails to account for the 2.5 moles of oxygen consumed per mole of metmyoglobin formed. Keilin (1961) found no evidence of hydrogen peroxide formation during hemoglobin autoxidation.

George and Stratmann (1954) found that the rate of myoglobin autoxidation in air increases approximately fourfold for a decrease in pH from 6.44 to 5.33. The same authors (1952b) reported that myoglobin autoxidizes 4.25 times faster in air than hemoglobin. Prior to all their kinetic measurements, George and Stratmann (1952a, 1952b, 1954) added a small quantity of dithionite (hydrosulfite) to convert the metmyoglobin formed during sample preparation to reduced myoglobin.

Snyder and Ayres (1961) studied the autoxidation in air of crystallized beef muscle myoglobin. The reaction was found to be first order in percentage oxymyoglobin and to have a mean rate constant value of 0.21 $\pm$ 0.02 $hour^{-1}$ when measured spectrophotometrically in 0.6 M phosphate buffer at pH 5.7 and 30°C. The rate constant was found to be dependent upon the amount of dithionite added at the beginning of the experiment. When large amounts (0.1 to 0.2 percent) of dithionite were used, the plot of log percent oxymyoglobin versus time showed 2 distinct phases, an initial steep slope followed by the characteristic, more gentle slope from

which the rate constants were calculated. The initial rapid rate of autoxidation disappeared when catalase was added, and consequently, was attributed to the presence of hydrogen peroxide. Presumably, the reaction of excess dithionite with oxygen produces hydrogen peroxide (Conant and Scott, 1926). Snyder and Ayres (1961) offered no explanation for the variation of rate constant with the amount of dithionite used. The same authors also studied beef myoglobin autoxidation at temperatures ranging from 0°C to 40°C and noted an unusually high temperature dependence. The autoxidation was characterized by an activation energy of 23,700 calories and a Q_{10} value of 4.8. The majority of enzyme systems are characterized by a Q_{10} value of 3 or less (West and Todd, 1957).

The autoxidation in air of beef and tuna myoglobin purified by diethylaminoethyl cellulose chromatography was studied by Brown and Dolev (1963a, 1963b). In the first paper, the authors (1963a) determined the rates of autoxidation at pH 5.9 in 0.1, 0.2, and 0.6 M phosphate buffer at temperatures ranging from 0 to 40°C. The autoxidation was found to be first order with respect to percentage oxymyoglobin under all conditions employed. The rates of oxidation of tuna myoglobin were independent of buffer concentration, whereas those of beef myoglobin decreased with increasing buffer concentration. Tuna myoglobin was oxidized more slowly than beef except under the combined conditions of high temperature and high buffer concentration. The actual rate constant values obtained by Brown and Dolev (1963a) for the autoxidation of beef myoglobin were appreciably less than those reported by Snyder and Ayres (1961) for the same conditions of phosphate concentration and temperature. Brown and Dolev

(1963a) assumed that the slightly higher pH of their test solutions accounted for the lower rate constant values they obtained. The same authors (1963a) also studied the autoxidation of a crude beef myoglobin preparation (beef muscle extracted with water and the extract dialyzed against several changes of water). The crude myoglobin preparation was oxidized more slowly than the purified beef myoglobin.

Brown and Dolev (1963b) studied the effect of freezing on the autoxidation of purified and crude preparations of both tuna and beef myoglobin. The autoxidation rates decreased with lower temperatures until the solutions solidified. Upon solidification, the rates increased sharply to maximum values at temperatures of -10 to -15°C. Repeated freezing and thawing did not greatly affect the rates of autoxidation. The authors (1963b) presumed that the increased rates of autoxidation obtained at sub-freezing temperatures reflected the establishment of a more suitable myoglobin-oxygen environment.

Recently, Snyder (1963) in a preliminary communication reported evidence for the dissociation of the heme group from myoglobin while undergoing autoxidation. A solution of beef metmyoglobin buffered at pH 6.6 was divided into 2 portions. After dithionite was added to one of the portions, acetone was immediately added to both portions which were then centrifuged. Analysis of the acetone extracts showed that 40 percent of the heme was removed from the reduced myoglobin and only a negligible amount was extracted from the metmyoglobin. Since the presence of free heme cannot be demonstrated after dialysis, electrophoresis, or column chromatography of aqueous solutions of myoglobin, Snyder (1963) postulated that the heme of reduced myoglobin remains physically asso-

ciated with the apoprotein although chemically dissociated. Based on the acetone extraction results, Snyder (1963) proposed that myoglobin autoxidation could possibly involve the following sequence of reactions:

$$MbO_2 \rightleftharpoons Mb + O_2$$

$$Mb \rightleftharpoons \text{heme} + \text{protein}$$

$$\text{heme} + O_2 \rightleftharpoons \text{hematin}$$

$$\text{hematin} + \text{protein} \rightleftharpoons \text{Met Mb}$$

where hematin represents ferric heme.

It has been well established (Rumen, 1959; Smith and Gibson, 1959; O'Hagan and George, 1960; Akeson and Theorell, 1960) that hematin and apoprotein, separated from metmyoglobin by acetone - HCl washing, can be recombined to yield a product having identical properties to the original metmyoglobin. The reversible splitting of myoglobin after treatment with dithionite has not yet been studied.

Multiple Occurrence of Myoglobins

Recent publications on the heterogeneity of human (Huisman and Dozy, 1962) and bovine myoglobin (Crockett et al., 1963) have indicated that a great deal of work has been performed in isolating and characterizing this multiple occurring protein. Various theories of hemoglobin type inheritance have been advanced (Crockett et al., 1963). In some cases, electrophoretic differences have been accounted for by the replacement of a single amino acid residue by another amino acid (Ingram, 1957).

The multiple occurrence of myoglobin has not been as fully explored as that of hemoglobin. Rossi-Fanelli and Antonini (1956) found 3 elec-

trophoretically (on paper, ionic strength of 0.01 - 0.03, pH 8.6) distinct myoglobin fractions, both in solutions of crystallized human myoglobin and in water extracts of human muscle. They termed these fractions myoglobins I, II, and III and showed that they occurred in the relative percentages 70-80, 10-20, and 5-7, respectively. In a later paper, Rossi-Fanelli and Antonini (1958) reported isolation of myoglobins I and II by electrophoresis and demonstrated their close similarity, both as to the absorption spectra of their respective reduced, oxy-, carboxy-, and met- forms and also as to their oxygen equilibrium properties. The 2 myoglobins presented oxygen dissociation curves at different temperatures, which were exactly alike in hyperbolic shape, high oxygen affinity, absence of the Bohr effect, and constancy with varying ionic strength and myoglobin concentration.

As quoted by Rossi-Fanelli et al. (1960), Rossi-Fanelli and Antonini in 1956 detected the presence of 3 myoglobin components both in crystalline preparations and in water extracts of tuna (Thunnus thynnus) muscle. The components, accounting for 80, 20, and 5 percent of the total pigment, had identical absorption spectra in the visible and Soret regions.

Rumen (1959) reported isolation of 5 myoglobins from seal (Phoca vitulina) muscle by chromatography on carboxymethyl cellulose with 0.01 M tris buffer at pH 8.5. The 5 myoglobins, which were termed myoglobins I, II, III, IV, and V in reverse order of their elution from the column, yielded 90, 2.5, 1.7, 1.7, and 3.4 percent, respectively. The homogeneity of the isolated myoglobins was demonstrated by moving boundary electrophoresis in phosphate buffer, ionic strength of 0.1, at various pH's in

the range 6.1-9.0. Rumen (1959) found the isoelectric point to be near pH 8.05 for myoglobin I and pH 7.6 for myoglobin II. The minimal molecular weight deduced from the iron content (0.29-0.30%) was found to be 18,600 for all 5 myoglobins. Spectrophotometric analysis of the met- and carboxy- derivatives of the 5 myoglobins showed them to have maxima and minima absorption at the same wavelengths, but to differ in extinction coefficients at several wavelengths. Glycine was found to be the N-terminal amino acid in all 5 myoglobins.

Akeson and Theorell (1960) isolated 3 myoglobins from horse muscle. On column chromatography using carboxymethyl cellulose (CMC) and 0.02 M phosphate buffer at pH 6.9, 2 myoglobin fractions were obtained. The slowest moving fraction was termed myoglobin I and the other fraction myoglobin II. Myoglobin I was purified by rechromatography at pH 6.4. Myoglobin II was completely separated from myoglobin I on rechromatography at pH 6.4 and then chromatographed on a diethylaminoethyl cellulose (DEAE) column with 0.02 M tris buffer at pH 8.4. On the DEAE column, myoglobin II separated into 2 myoglobin components, myoglobin II_1 and II_2. Myoglobins I, II_1, and II_2 were tested for homogeneity by moving boundary electrophoresis at pH 6.0, 8.0, and 9.8. Akeson and Theorell (1960) found that the sulfur and iron contents were the same in all 3 myoglobins, with the iron content giving a minimum molecular weight of 18,500. No significant difference was found in the amino acid compositions or in the amide nitrogen contents of the 3 myoglobins. The absorption spectra were identical for both the carboxy- and met- derivatives of the 3 myoglobins over the range 380-1000 mu, and showed only slight differences in the 235-380

mu region. Mixtures of the 3 myoglobins in varying proportions sedimented homogeneously in the ultracentrifuge.

Akeson and Theorell (1960) did, however, find a primary structural difference among the 3 myoglobins. The individual myoglobins were split by treatment with acidified acetone and their respective apoproteins were isolated and subjected to tryptic digestion. The peptides resulting from apomyoglobin digestion were separated on paper. The paper was spotted with a digestion mixture and submitted first to horizontal electrophoresis and then to descending chromatography. Two specific peptides were discovered. Both were missing in myoglobin II_2, and both were present in myoglobin I. Myoglobin II_2 contained one of the specific peptides.

Edmunson and Hirs (1961) fractionated the carboxy-derivative of crystalline sperm whale myoglobin into 5 components by column chromatography using Amberlite IRC-50 and citrate buffer (0.34 N in sodium ion) at pH 5.9. Components I, II, and III accounted for 2-3, 7-10, and 20 percent, respectively, of the total heme protein, while components IV and V together accounted for the remainder. Quantitative amino acid analyses were performed and found to be the same, within the range of experimental error, for components II, III, IV + V, and V. Component I was thought to be contaminated by colorless protein(s).

In a recent paper, Perkoff et al. (1962) implied criticism of earlier work performed on myoglobin heterogeneity. They separated human myoglobin into 4 heme fractions (F_1, F_2, F_3, F_B) on DEAE cellulose using tris buffer at pH 7.8, and applying either temperature change or sodium chloride concentration to effect gradient elution. Fraction F_3 was heterogen-

eous and contained some non-heme protein. Fractions F_1, F_2 and F_B were suggested to differ only in their prosthetic groups. The prosthetic group of F_B was considered to be a heme degradation product. F_1 and F_2 were reported as differing only in the state of the heme iron, F_1 being acid metmyoglobin and F_2 being alkaline metmyoglobin. Evidence for the chromatographic resolution of the acid and alkaline forms of metmyoglobin consisted of a comparison of the spectral curves of F_1 and F_2 with spectra of the acid and alkaline forms reported in the literature, and also of the fact that the cyanide derivatives of F_1 and F_2 exhibited exactly the same chromatographic and starch gel electrophoretic behavior.

The partial explanation of heterogeneity, based on differences in the state of the iron, could be applicable to the results of Rossi-Fanelli and Antonini (1956, 1958), Rossi-Fanelli et al. (1960) and Rumen (1959), but would not be pertinent to the findings of Edmunson and Hirs (1961), because of the low pH of their chromatographic separation. Similarily, Akeson and Theorell (1960) demonstrated 2 myoglobin components by chromatography at a pH at which the alkaline form of metmyoglobin would not be expected to exist. These last authors also demonstrated a primary structural difference among the myoglobins.

Lewis and Schweigert (1955) demonstrated heterogeneity of crystalline beef muscle myoglobin by moving boundary electrophoresis in phosphate buffer at pH 8.0. Besides the main component, 2 substances, A and B, accounting for 15-20 and approximately one percent, respectively, of the total protein were detected. Substance A was isolated and studied. Its absorption spectra resembled that of myoglobin. Acid-cleavage data,

however, indicated to the authors that the heme-protein linkages were different in substance A and myoglobin. The heterogeneity of the crystalline myoglobin could not be demonstrated by ultracentrifugation.

EXPERIMENTAL PROCEDURE

Preparation of Crude Myoglobin

Extraction and Concentration

Cow rounds were purchased locally, boned and trimmed of visible fat. The lean meat was ground and packed in 10 lb. lots into cryovac bags. The bags were then evacuated, frozen and placed in frozen storage at -20°F. The meat was removed as needed from the freezer and thawed at room temperature. It was homogenized in a Waring blendor for 30-50 seconds with approximately an equal weight of distilled water. The thick slurry was then frozen at -20°F and thawed at room temperature. As the frozen slurry thawed, the drip was filtered through gauze and collected. The freezing-thawing procedure was found to be the easiest and cleanest method for separation of the myoglobin fraction from the homogenate. The almost colorless residue contained an ice lattice, even after most of the drip had been collected. The drip volume was reduced from approximately 7 liters to less than one liter by pervaporation in cellulose dialysis tubing at room temperature. One fan provided a sufficient rate of evaporation to maintain the temperature of the drip at 14-16°C.

Concentrating the drip by repeatedly freezing and thawing as suggested by Fox (1961) was also tested. This method, although effective, proved too time-comsuming.

The concentrated drip was centrifuged at 2500 rpm for several hours and filtered through coarse filter paper to remove proteins precipitated

during pervaporation. The gummy precipitate could not be completely sedimented even after prolonged centrifugation at 2500 rpm and clogged up the filter paper. The addition of celite provided a somewhat faster filtering rate.

Ammonium sulfate fractionation

The procedure of Lewis and Schweigert (1955) was used with modification The fractionation procedure is based on the insolubility of myoglobin at its isoelectric point of pH 6.9-7.0 at approximately 80 percent ammonium sulfate (Am SO_4) saturation. The filtrate was taken to 65 percent saturation with solid Am SO_4. Ammonium hydroxide was added to keep the pH above 6. The viscous precipitate at 65 percent saturation was removed as previously described. The filtrate was then taken to complete Am SO_4 saturation and the precipitate containing the myoglobin fraction was collected in the centrifuge. This precipitate was dissolved in a small amount of deionized water and dialyzed against a 75 percent Am SO_4 saturated solution of pH 7.0. The supernatant containing the crudely purified myoglobin was taken to 90 percent saturation with solid Am SO_4 and stored at 0°F until needed for chromatography.

Heating of the concentrated drip at 55°C for 5 min., according to the method of Snyder and Ayres (1961), resulted in faster and much more complete centrifugal sedimentation of the precipitates encountered after pervaporation and the first salt fractionation. No apparent destruction of the myoglobin occurred during the heating process, if the pH of the

concentrated drip was first increased to above 6. The heat treatment was incorporated into the crude purification procedure and used throughout the latter portion of this study.

All centrifugation and salt fractionation was carried out at 3-5°C. Meat from 5 different animals totaling approximately 100 lbs. was used during the course of these experiments.

Sephadex column chromatography

The use of Sephadex molecular sieve chromatography in preparing myoglobin of a purity equivalent to that of the crystalline protein was suggested by Fox (1961). Dry Sephadex G 75 was stirred into a solution of 0.1 M triethanolamine (TEA) and 0.2 M NaCl at pH 7.0. After the finest gel particles were removed by twice-repeated sedimentation and decantation, the suspension was transferred to a 2 liter separatory funnel mounted above a chromatographic column, which was filled with the buffer-NaCl solution. After a 3-5 cm layer of gel grains had settled at the bottom of the column, the stopcock was opened to allow a slow flow of the buffer-NaCl solution. When all the grains had settled, several thicknesses of filter paper were placed on top of the gel bed. Five 2.5 x 55 cm columns and one 6.5 x 90 cm column were built in this manner.

The crude myoglobin precipitate in 90 percent Am SO_4 was removed from 0°F storage and collected by centrifugation. It was dissolved in a small volume of the TEA - NaCl solution and then dialyzed against the buffer until free of sulfate. Sulfate in the dialyzate was detected by

the formation of a white precipitate upon addition of barium chloride. During dialysis the myoglobin had to again be concentrated by pervaporation; the high concentration of Am SO_4 within the dialyzing membrane causing a large influx of water. After dialysis, the myoglobin sample, which contained approximately 15 mg. of myoglobin per ml., was placed on the column and washed in with 2 small quantities of the TEA - NaCl buffer. The same buffer was used in elution.

The volume of sample placed on the 2.5 x 55 cm columns ranged from 15 ml to 40 ml. The flow rate of the small column was fairly slow (about 5 mls per hour) and could not be increased by adjusting hydrostatic pressure. The flow rate of the large 6.5 x 90 cm column was so slow that it was discarded. The eluent was collected in 10 or 20 ml fractions and read spectrophotometrically at 280 mu and 525 mu. Several eluents were also read at 410 mu. An absorbancy ratio was calculated by dividing the absorbancy at 280 mu by the absorbancy at 525 mu. Fractions having a ratio value of less than 4.5 were retained.

Crystallization

The crystallization method of Lewis and Schweigert (1955) was originally tested. The method involves the addition of solid Am SO_4 to a previously purified and concentrated myoglobin preparation until 80 percent saturation is reached. Dropwise addition of a saturated solution of Am SO_4 and centrifugal removal of amorphorous precipitates is then continued until myoglobin crystallizes. The pH of the preparation is

maintained at 6.7 and the temperature at 3-4°C. This procedure proved unsuccessful in the hands of the author.

The method of Luginbuhl (1961) who crystallized human myoglobin, was also tested and likewise proved unsuccessful. This method is based on the solubility of human myoglobin in saturated Am SO_4 at pH 8. After removal of the hemoglobin containing precipitates under these conditions, the pH is adjusted to 7 and the human myoglobin crystallizes. It was found that very little beef myoglobin remained in solution at pH 8 after complete Am SO_4 saturation.

Crystals were finally obtained from Sephadex purified myoglobin. A Sephadex eluent having a 280 mu/525 mu absorbancy ratio of less than 4.5 was concentrated by precipitation with Am SO_4. The precipitate was dissolved in 70 percent Am SO_4 and dialyzed against a 75 percent (480 g/l) Am SO_4 solution of pH 6.9-7.0. The small amount of precipitate formed was removed by centrifugation and the supernatant was placed in a centrifuge tube. Saturated Am So_4 solution was added dropwise until cloudiness in the tube was observed. After standing for several hours the precipitate formed was removed by centrifugation, washed with saturated Am SO_4 solution, and examined under a microscope. The dropwise addition and removal of precipitates formed was continued until myoglobin crystallized.

Electrophoretic Detection of Multiple Myoglobins

Electrophoresis on paper and cellulose acetate

The myoglobin eluent from a Sephadex column was divided into 2 fractions. One fraction had a 280 mu/525 mu absorbancy ratio value of less

than 4.5, while the other had a ratio value of 4.5 to 5.0. Samples of each were dialyzed against either veronal or borate buffer.

The samples were tested for heterogeneity on Whatman 3 MM paper strips in a hanging strip, inverted-V, Durrum-type electrophoretic cell assembly. Current was supplied by an adjustable, constant voltage, DC power supply. Veronal and borate buffers at pH 8.6 and at ionic strengths of both 0.050 and 0.075 served as the electrolyte. The voltage was maintained at 200 volts. The current gradually decreased during a 7 hour run from 5-7 ma. to approximately 2 ma. After electrophoresis, the paper strips were dried and stained with bromphenol blue dye solution. Electrophoresis was performed both at 4°C and at room temperature. The heterogeneity of beef myoglobin could not be demonstrated under any of these conditions. Indeed, the furthest the single protein band moved even after 16 hours was less than one inch.

Cellulose acetate strips have an advantage over paper in that they possess minimal adsorption properties. Oxoid cellulose acetate strips were obtained and cut in lengths to allow their accommodation in the electrophoretic cell. Electrophoresis was carried out at 150-200 volts and at 0.4 ma. per cm. of strip width. The same buffer conditions as employed for the paper electrophoresis were applied at 4°C. After electrophoresis, the strips were dried and stained with either of the protein dyes, Ponceau S or the very sensitive Nigrosin. The cellulose acetate strips and the 2 dyes were obtained from Consolidated Laboratories, Chicago Heights, Illinois. Although a 4 hour electrophoretic run produced a migration of approximately 5 cm. no evidence of heterogeneity was demon-

stratable in either of the Sephadex eluent fractions. When mixed together the 2 fractions migrated as one.

Moving boundary electrophoresis

Sephadex-purified myoglobin (250 mu/525 mu value of less than 4.5) was concentrated to approximately one percent protein by pervaporation and dialyzed against veronal buffer of pH 8.7 at an ionic strength of 0.1. Electrophoresis in the veronal buffer was performed in a Perkin Elmer 38A instrument at approximately 1°C and at 10 ma. Only a single peak was detected after 63.3 minutes.

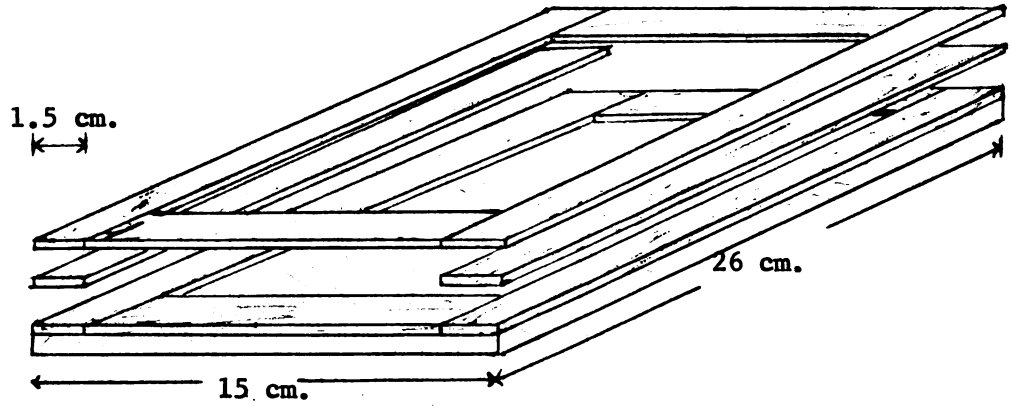
Starch gel electrophoresis

The use of gel made from hydrolyzed starch as a carrier for zone electrophoresis was initiated by Smithies (1955), who demonstrated its resolving power with human serum proteins to be considerably greater than any other electrophoretic procedure.

Starch gels were prepared by the method described by Smithies (1955). Dry, hydrolyzed starch was obtained from Connaught Medical Research Laboratories, Toronto. To prepare a gel, 35.4 gm. of the dry starch was suspended in 300 ml. of buffer in a 1000 ml. beaker. Continuous rapid stirring was carried out as the suspension was heated over a Bunsen burner. As the temperature increased, the suspension first turned semi-solid and then became a viscous fluid. When the temperature reached 90°C, the beaker was placed in a dessicator attached to a vacuum pump. To remove the gas from the suspension, negative pressure was applied until the hot fluid boiled vigorously and bubbles were observed rising from the very

bottom of the beaker (approximately 5 sec.). The boiling time was kept short so that water loss would not appreciably affect the composition of the gel. The hot fluid was then poured into the electrophoretic cell assembly, so as to completely fill it. A slot-former was placed carefully in position about 6 cm. from one end of the assembly while avoiding the formation of air bubbles. The slot-former consisted of a piece of 15 by 2 cm. plexiglass onto which 6 pieces of 2.5 by 6 by 8 mm. plexiglass were fused in a row. When the slot-former was placed in position, the 6 pieces dipped into the hot fluid to a depth of 6 mm. After insertion of the slot-former, the gel was placed in an atmosphere saturated with water vapor. After cooling to room temperature (2-3 hours), the gel was ready for use.

The electrophoretic cell assembly was of the form described by Wake and Baldwin (1961). It consisted of a plexiglass tray, the sides of which were made from 3 sections, each 2.5 mm. in depth. The lowest section was fused on a sheet of plexiglass to form the base of the cell.



Disassembled starch gel tray.

The 2 removable top sections were fixed in position on the base by fastening with tape along the sides and at the corners. The assembled tray presented a narrow slit opening at each end. Before pouring the gel, heavy chromatographic paper approximately 15 cm. in length and of a width cut to tightly fit the openings was inserted into each opening to a depth of approximately 3 cm.

The tray containing the cooled gel was suspended horizontally between 2 electrode vessels. The heavy chromatographic paper dipped into the electrolyte solution and served as electrolyte bridges. The electrode vessels were constructed of 4 mm. thick plexiglass and were of a size to allow the accommodation of 2 cell assemblies. Each electrode vessel had a capacity of 6 liters of solution. The vessels were connected by tygon tubing in order to equalize their solution volumes after an electrophoretic run. The tubing was clamped off during a run. The electrodes consisted of 25 cm. of platinum wire stretched across the vessels. The electrodes were connected to an adjustable, constant voltage, DC power supply to complete the electrophoretic circuit.

All starch gel electrophoretic runs were performed at 3-4°C. To minimize evaporation, the gels were covered with a layer of mineral oil and the complete tray and the exposed paper bridges were then covered with Saran wrap. The gels were placed in position approximately 1/2 hour before being used.

The slot-former was carefully removed from the gel and the myoglobin samples, which had previously been equilibrated with the buffer in which

the gel was made, were inserted with a capillary tube. The filled slots were then covered with mineral oil. After a few minutes, the current was applied. The maximum voltage applied was 200 volts and the maximum current was 25 ma. At 200 volts, the current decreased during a 12-14 hour run to about 10 ma.

After electrophoresis, the tape, and then the top section of the tray sides were removed from the cell assembly. The exposed 2-3 mm. of gel was sliced off with a thin (piano) wire tautly stretched in a coping saw frame and was discarded. The middle section of the tray was then removed and the second slice was made. This slice, which had a freshly cut surface on both sides, was then peeled off the remaining gel into the staining dish by inverting the tray over it.

Either a protein or a heme-detecting dye system was used in staining the gels. The protein-staining technique was that of Smithies (1955). Two g. of Amido-Black 10B dye were dissolved in a mixture of 250 ml. water, 250 ml. methanol and 50 ml. acetic acid. The dye solution was poured onto the gel and was removed after one minute. The gel was then rinsed with the water-methanol-acetic acid mixture until the gel background was free of blue stain. The dye solution was re-used 20-30 times. The heme-detecting method, which was used in this study, is based on the peroxidase activity of heme groups. The technique of Owen et al. (1958) was employed. One hundred mg. of o-dianisidine (3,3'dimethoxybenzidine) was dissolved in a mixture of 70 ml. ethanol, 18 ml. water and 10 ml. of 1.5 M acetate buffer at pH 4.7. Immediately before use, 2 ml. of 3% hydrogen peroxide were added. The staining solution was placed on the gel slice for 15 to 30 minutes. The staining time depended upon the observable color intensity

of the reddish-brown stains formed. The gel was then rinsed several times with water. The peroxidative staining method was found to be much more sensitive for the detection of heme-containing proteins than the Amido-Black method. The gels were stored in their rinsing solutions but could not be preserved indefinitely as they became very fragile with time.

Initially, the gels were made with 7.0 M urea as suggested by Wake and Baldwin (1961), who worked with the casein proteins. The urea was added at approximately 65°C. during the heating of the starch suspension. The urea-starch gels were superior to non-urea gels, in that they resulted in more sharply defined separation (less tailing), were more transparent, were more resistant to tearing on subsequent handling during slicing and staining, and could be dried and kept indefinitely as permanent records. However, it was noted that the spectrum of myoglobin dialyzed against buffered 7 M urea was markedly different from that of native myoglobin. Myoglobin samples separated into twice as many protein-staining zones on urea gels as on non-urea gels, but into the same number of heme-staining zones. This evidence of myoglobin denaturation by urea excluded further useage.

Several buffer systems were tested with both urea and non-urea gels. The borate buffer of Smithies (1955) was used both in the preparation of the gel and as the electrolyte. This buffer contained 0.022 M boric acid and 0.009 M NaOH and had a pH of 8.6. Sigma, grade 121, tris (2-amino-2 hydroxymethyl-1,3 propanediol) buffer was also used as the electrolyte and in the gel preparation. Portions of a 0.76 M tris, 0.05 citric acid stock buffer of pH 8.9 at 4°C were diluted to 10 volumes for use. Neither of these buffers, serving alone, resulted in as satisfactory a myoglobin separation as the discontinuous buffer system devised by Poulik (1957). By this technique, the gels were made with the diluted

tris-citrate buffer solution, but the electrode vessels and connecting paper bridges contained a 0.30 M boric acid -0.06 M NaOH buffer. According to Smithies (1959), during electrophoresis a boundary is formed between the 2 buffers and is associated with a very abrupt change in voltage gradient, which sharpens many of the zones. This buffer system resulted in distinct separation of 3 heme- and protein-staining zones and was subsequently employed in the electrophoretic testing for the purity of chromatographic eluent fractions.

Several electrophoretic experiments were performed to eliminate the possibility of myoglobin separation due to differences in the state of the iron. The myoglobin samples were oxidized with potassium ferricyanide and dialyzed against tris-citrate buffer containing 0.001 M potassium cyanide. The gels and the borate buffer also contained 0.001 M KCN. The electrophoretic patterns of the cyanide derivatives were compared to those of the normal myoglobin samples.

Separation and Isolation of Multiple Myoglobins

Carboxymethyl cellulose chromatography

A quantity of the ion-exchange adsorbent, carboxymethyl (CM) cellulose, was prepared by the method of Peterson and Sober (1956). A solution of 90 g. NaOH in 200 ml. water was stirred into 60 g. of Whatman cellulose powder (standard grade) in a 2 liter Erlenmeyer flask. The flask was immersed in ice water for 30 min. and the contents were stirred occasionally. A solution of 30 g. chloroacetic acid in 40 ml. water was added in 4 portions and mixed well after each addition. The soft, dough-like mass was then heated in a 70°C bath for 20 min. with occasional

stirring. The flask was cooled in an ice bath, and 500 ml. of 10 percent acetic acid were added in 4 portions. The suspension was then diluted to 2 liters with water and allowed to settle. After decantation of the yellow, cloudy supernatant, the sediment was washed 5 times with water in a similar manner. Glacial acetic acid (about 10 ml.) was then added to the diluted suspension and the pH was adjusted to between 3 and 4. After settling and decantation, the sediment was washed with water until it was no longer acid. A slurry of the sediment was then poured into a Büchner funnel equipped with coarse filter paper (milk filter discs) and dried by vacuum. The cake was washed twice with ethanol and again dried by vacuum. About 30 g. of white powder was obtained. The carboxyl content of the modified cellulose was determined by titration with HCl after the addition of a small excess of NaOH and was found to be 0.45 meq. per g. of dry powder.

Twenty-five g. of the CM cellulose powder were equilibrated with the 0.02 M, pH 6.90 phosphate buffer employed by Akeson and Theorell (1960). Equilibration was attained by stirring the cellulose in repeated changes of the buffer until the pH of the suspension reached 6.90. A chromatographic glass tube of 4.1 cm. internal diameter by 25 cm. in length was fitted with glass wool and half-filled with buffer. The equilibrated CM cellulose in the form of a thick slurry was added in 5-6 portions. The suspension was allowed to settle by gravity while the buffer slowly flowed through the column. Air pressure was then applied to compress the sediment to a heighth of 16 cm. Finally, several layers of filter paper, cut to tightly fit the internal diameter, were placed on top of the column bed.

Sephadex-purified myoglobin samples were pervaporated to a concentration of 20-25 mg. per ml. and dialyzed against the pH 6.9 phosphate buffer until free of chloride ion. The presence of chloride in the dialyzate was detected by the appearance of a white precipitate upon adding silver nitrate. A small quantity of potassium ferricyanide was then added to the dialyzate to oxidize any remaining oxymyoglobin in the sample, and dialysis was continued against 2 or 3 more changes of buffer. Ten to 25 ml. aliquots of the samples were placed on the column, washed in with 2 small portions of buffer and eluted with the same buffer. The flow rate was adjusted for different experiments over a range of 0.4 to 4.0 ml. per minute. The eluent was collected in 10 ml. fractions, and the fractions were read spectrophotometrically at 280 and 525 mu. On the basis of spectrophotometric analysis, the eluent was divided into 2 large fractions. These fractions were labelled Mb I and Mb II in the order of emergence from the column. The 2 fractions were concentrated to volume and dialyzed against the buffer to be used in subsequent chromatographic experiments.

Fractions Mb I and Mb II were re-chromatographed on CM cellulose columns using both 0.02 M phosphate buffer at pH 6.40 according to the procedure of Akeson and Theorell (1960), and 0.01 M tris buffer at pH 8.50 according to the procedure of Rumen (1959). The CM cellulose used in these and following experiments was Whatman CM 70 powder, which contained 0.7 meq. acidic groups per g. dry weight. Chromatography at either pH 6.40 or pH 8.50 did not further fractionate or purify either Mb I or Mb II, but only diluted them.

The use of Sephadex chromatography in the purification procedure was discontinued at this point. The crude myoglobin preparation in 90 percent Am SO_4 was removed from 0°F storage, collected by centrifugation and dialyzed against the 0.02 M, pH 6.9 phosphate buffer until free of sulfate. The presence of $AmSO_4$ in the dialyzate was detected by spot-testing for the ammonium ion according to the method published by Feigl (1958). P-nitrobenzenediazonium reagent was prepared by reacting p-nitraniline with sodium nitrate in dilute hydrochloric acid. A drop of the dialyzate was placed on a spot plate followed by a drop of the reagent and then by a particle of calcium oxide. The formation of a red color around the particle of calcium oxide indicated the presence of ammonium ion. The spot test was used in place of the barium chloride test previously mentioned because both phosphate and sulfate give a white precipitate with barium.

After removal of $AmSO_4$, the crude myoglobin preparation was pervaporated to volume and oxidized with ferricyanide as previously described. It was then dialyzed against several more changes of the phosphate buffer.

Whatman CM cellulose, equilibrated with 0.02 M, pH 6.9 phosphate buffer was used to build a column bed 4.7 by 24 cm. The building technique was the same as that previously reported for the CM cellulose prepared in this laboratory except that 7 cm. of glass beads were placed on the filter paper over the top of the bed. The weight of the beads served to keep the resilient bed compressed after the air pressure had been released. All subsequent CM cellulose chromatography was performed on this same column bed.

Experiments were performed to determine the optimal pH and protein load conditions for resolution of Mb I and Mb II. The pH was varied from

6.82 to 7.20 and the sample, containing 25 mg. myoglobin per ml., was applied in volumes ranging from 40 to 100 ml. It was found that resolution of Mb I and Mb II on Whatman CM cellulose in 0.02 M phosphate buffer was optimal and independent of pH within the range 6.85 to 6.95. Eighty ml. of sample could be applied without loss of resolution.

During the final stages of this study, sample dialysis and chromatography were carried out in the phosphate buffer containing 0.001 M potassium cyanide. All other conditions were the same as those previously described. Nevertheless, Mb I and Mb II emerged from the column overlapping to the extent that they could not be treated as distinct fractions.

Diluting the phosphate concentration in the buffer to 0.01 M, however, resulted in better separations than those hitherto attained. The pH of the diluted buffer could be increased to 7.05 without loss of resolution.

When satisfactorily separated by any of the conditions described, pooled eluent fractions of Mb I and Mb II were concentrated by pervaporation and dialyzed against the buffer used in subsequent diethylaminoethyl (DEAE) cellulose chromatography.

Diethylaminoethyl cellulose chromatography

Whatman DEAE (DE 50) cellulose containing 1.0 meq. basic groups per g. dry weight was equilibrated with 0.02 M tris buffer titrated to pH 8.3 with 2 N HCl. A column bed 2.7 by 50 cm. was built in the manner previously described. The column bed was air-compressed to the extent that a period of 5-7 hours was required to load it with a 10 ml. sample under gravity-flow conditions. The flow rate of the developing column could be controlled by adjusting the hydrostatic head pressure. A 3-4 liter volume

of buffer suspended 4-5 ft. above the column induced a flow rate of approximately 1/2 ml. per minute. Initially, Eastman DEAE cellulose was used for the columns. However, this material packed so tightly that column beds exceeding 25 cm. in heighth would not allow a sufficiently rapid chromatographic development.

After approximately 100 ml. buffer had passed through the column containing either Mb I or Mb II, a brown-colored band began to move down the bed, broadening as it moved. After it had moved to within a few cm. of the bottom of the column, the whole column bed was pushed out of the glass tube by gentle application of compressed air. The broad band at the base of the column and the band that remained at the top were cut off from the rest of the cellulose bed. In order to extract the myoglobin from the cellulose, the bands were stirred in 0.02 M phosphate buffer at pH 6.9 and centrifuged. The extraction procedure was repeated until the cellulose was free of color. The pooled supernatants of the 2 bands were then tested for homogeneity by starch gel electrophoresis.

Unsatisfactory electrophoretic results prompted further DEAE chromatographic experiments. Chromatographic trials in 0.02 M tris buffer at both pH 8.00 and pH 7.83 were performed. At either pH value both Mb I and Mb II were further chromatographically fractionated. On cellulose beds of 2.7 by 50-55 cm., Mb I separated into 6 and Mb II into 4 distinct, colored bands. The bands were extracted in the manner previously described and subjected to electrophoretic analysis. None of the bands proved homogeneous. Each band contained all 3 of the electrophoretically-detectable myoglobins in varying proportions. Consequently, all chromatographic procedures based on complete elution with a single buffer were abandoned.

Stepwise column-development techniques were then tested; i.e., a series

of buffers of successively greater eluting power were applied to the columns.

Columns of DEAE cellulose equilibrated with 0.02 M tris buffer at pH 8.62 were built in the manner previously described. Samples were equilibrated and washed into the columns with the same buffer. It was not necessary to extensively concentrate samples before loading columns equilibrated with 0.02 M tris at any pH above 8.4. Sample volumes of up to 100 ml. could be used to load column beds of 2.7 cm. in diameter. The protein was concentrated and retained in a single band at the top of the bed while the buffer in which it was loaded flowed through the columns. The column was then developed using 4 successive 100 ml. volumes of 0.02 M tris buffer, either adjusted to successively lower pH values (8.30, 8.03, 7.64 and 7.01) or containing increasing molarities of NaCl (0.01, 0.05, 0.10, 0.20). However, neither of these development techniques resulted in satisfactory myoglobin fractionation as determined by electrophoretic analysis.

At this point, 0.001 M potassium cyanide was added to all dialyzing and chromatographic buffers. Chromatography at pH 8.01 using the same 0.02 M tris buffer throughout complete elution was repeated. While the KCN treatment resulted in the disappearance of 2 of the 6 Mb I bands previously found, the remaining 4 bands of Mb I and the 4 bands of Mb II were as electrophoretically heterogeneous as before.

The following chromatographic procedure, involving a stepwise elution technique, resulted in the most homogeneous separation of the 3 electrophoretically-detectable myoglobins yet attained. DEAE ceullose was

equilibrated with 0.005 M tris buffer at pH 8.42 and packed into chromatographic tubes. Slight modifications in the column-building technique had been evolved during the course of this study. The equilibrated DEAE cellulose slurry was added in several portions to the chromatographic tube half-filled with buffer and allowed to settle as before. Air pressure was applied after each addition. After air-compressing the last portion of slurry, several layers of stiff, coarse filter paper (milk filter discs) were placed on the column bed, followed by several layers of fine filter paper and then by 2 or 3 layers of the coarse paper. The filter paper was cut to slightly exceed the internal diameter of the column, and thus had to be forced into the tube. The column bed was then further compressed by applying force on the filter paper by the use of a plunger. The plunger consisted of a hollow glass tube of an external diameter slightly less than the internal diameter of the chromatographic tube. Columns packed in this manner very seldom exhibited channeling. DEAE cellulose beds of 1.7 by 12-15 cm. were used. The smaller beds were of a sufficient heighth to produce resolution by the stepwise procedure, and at the same time, were more easily packed and allowed a faster flow rate. The myoglobin samples were equilibrated and washed into the columns with the 0.005 M tris buffer at pH 8.42. Mb I was then eluted with 0.02 M tris at pH 8.42. A buffer mixture containing 4 parts of the 0.02 M tris buffer and 1 part of the phosphate buffer (0.01 M, pH 6.9) that was used in CM cellulose chromatography was applied. A very narrow red-colored band moved down the column and was collected. The third colored fraction in a very narrow band was then eluted with the phosphate buffer alone.

The pH of the tris-phosphate buffer mixture was 8.30. All buffers contained 0.001 M KCN. The second and third bands were termed Mb I_A and Mb I_B in the order of emergence from the column since they occurred in greatest proportions in the CM cellulose fraction, Mb I.

The 3 DEAE cellulose fractions were dialyzed for 2 days against several changes of 0.6 M phosphate buffer at pH 5.8 in order to dissociate the cyanide and reconvert the myoglobin to its met-derivative. Prior to characterization analysis, the fractions were concentrated by pervaporation and read spectrophotometrically from 500 to 640 mu to make certain that no cyanmetmyoglobin was present.

Concentrating protein by pervaporation also resulted in concentrating the buffer salts present and necessitated further dialysis. For this reason, and also because of the esthetic inelegance of hanging a dialysis sac in front of a fan, the Sephadex method of concentrating protein according to the procedure of Flodin et al. (1960) was tested. Dry sephadex G 25 was stirred into the protein-containing solution and was then removed by centrifugation. The Sephadex, however, took up myoglobin as well as the buffer. Hence, the method was abandoned.

Techniques Used in Routine Analysis

Protein concentration

Initially, myoglobin concentrations were determined by the spectrophotometric absorbancy measurement of either the protein at 280 mu or the cyanide derivative at 540 mu. A salt-free, crudely-purified myoglobin sample was used in developing a standard curve relating absorbancy at 280 mu to protein concentration. The concentration of protein was determined by drying a known volume of the sample to a constant weight in an oven at 100°C.

A molar absorptivity value at 540 mu of 11.3 x 10^3 liters per mole cm. (Bowen, 1949) and a molecular weight of 17000 g. were used in estimating the concentration of myoglobin by the absorbancy of its cyanide derivative.

Both of these methods were employed in determination of protein concentrations for chromatography experiments. When preliminary spectral results indicated a substantial difference in absorptivity between the myoglobin fractions at 280 and 540 mu, the spectrophotometric method of Waddell (1956) was tested. The method is based upon protein absorption in the short wavelength region of the ultraviolet spectrum. Light absorption at these wavelengths is primarily a function of the peptide bond and is, therefore, relatively independent of amino acid compositions. All proteins should, then, exhibit relatively the same spectral characteristics in this wavelength region. Waddell (1956) subtracted the absorbancy at 225 mu from that at 215 mu and multiplied the difference by a derived factor (144) to obtain ug protein per ml. The method was developed using human serum albumen and globulin proteins and was found to be independent of the type of protein and of pH from 4 to 8. The applicability of the method in determining myoglobin content was tested by comparing it with the micro Kjeldahl technique on samples of Mb I_A and Mb II. It was found that the method could be applied using the factor of 144 derived by Waddell (1956). However, the absorbancy must not exceed a value of 1.40 as deviations from linearity occurred at higher absorbancies. The method was subsequently employed in all myoglobin characterization studies.

Measurement of pH.

The pH of all the buffers ultimately adapted for starch gel electrophoresis, and CM and DEAE cellulose chromatography, as well as the pH values of all characterization studies were determined with a Beckman, model G, pH meter. When pH values were determined to the second decimal point, the meter was calibrated using a commercial, standard buffer with a pH value within ± 0.3 units of the test solution. The standard buffer and the test solution were adjusted to the same temperature. All buffers were made at room temperature. However, final pH readings of the electrophoretic and chromatographic buffers were made at the temperature of use (3-4°C). This precaution was especially important in the case of tris buffers. It was found that the pH of tris buffers increased an average of 0.02-0.03 units per °C decrease in temperature. All pH readings were estimated to have an uncertainty of 0.02 units.

Spectrophotometry

A Beckman Model DU spectrophotometer equipped with photomultiplier attachment and DC power supply was used in preliminary experimentation. All final characterization results were obtained using the Gilford model 220 absorbance indicator (Wood and Gilford, 1961a) attached to the DU light source and monochromator. A Sola 10 kva transformer provided constant voltage (115 V). A Sargent model SR recorder was attached for use in reaction studies. When coupled with the Gilford model 210 automatic cuvette positioner (Wood and Gilford, 1961b) and the recorder the absorbance indicator permitted the direct absorbancy recording of 3 simultaneous reactions. Sargent brand, silica, rectangular cuvettes of 3 ml. capacity and 10 mm. light path were used in all studies.

Both the digital readout and recording systems were calibrated and checked periodically with Gilford absorbancy standards. The digital readout system was considered to give an absorbancy reading uncertainty of 0.002 units. The uncertainty of recorded values was estimated to be less than one percent.

Characterization of Multiple Myoglobins

Absorption spectra

Mb I_A, Mb I_B and Mb II were dialyzed against 0.05 M phosphate buffer at pH 6.90 and read spectrophotometrically at wavelengths between 725 and 214 mu. The myoglobin samples were diluted to approximately 1/3 their initial concentrations for reading below 450 mu.

The carboxy-derivatives of the 3 myoglobins were prepared by a modification of the method of Poel (1949). The deaminizing vessel from a Van Slyke amino nitrogen apparatus was attached to a vacuum line at the top and to a source of carbon monoxide at the bottom. The sample was placed in the reaction chamber and the complete system was evacuated. Carbon monoxide was then allowed to slowly bubble through the solution for 5 minutes. When the pressure within the vessel reached atmospheric, as judged by equal heights of the solution in the reaction chamber and in the connecting side arm to the reagent funnel, the stopcock to the reagent funnel was opened and a small amount of sodium dithionite in a known volume of the 0.05 M phosphate buffer was added. The system was again completely evacuated and carbon monoxide was bubbled through the solution for an additional 5-7 minutes. The carboxy-myoglobin was then allowed to

flow into a cuvette. The cuvette was quickly sealed with paraffin paper. The sample was then immediately read in the spectrophotometer at wavelength intervals from 650 to 400 mu. After taking the spectrum, the sample was again read at several wavelengths in the 550-630 mu region to determine whether autoxidation had occurred.

The absorbancy values of both the met- and carboxy-derivatives were converted to their respective molar absorptivity (E) values by assuming a molecular weight of 17,000 for each of the myoglobins. The E values were then plotted against wavelength.

Heme content

Either heme content or iron content may be used in comparing the molecular weights of the myoglobins. The relatively small amount of sample required for heme determination made it the method of choice. The procedure of Keilin and Hartree (1951) was used with minor modifications. Sample volumes of 1.0-1.5 ml. containing a known amount of protein were placed in a test tube. Pyridine, NaOH solution and then water were added to make the final volume 2.5 ml. and the final molarities of pyridine and NaOH 2.1 and 0.075, respectively. A few crystals of sodium dithionite were then added and the contents were thoroughly shaken and allowed to stand for 1/2 hour. The absorbancy of the bright red solution was measured at 557 mu and divided by the known concentration of protein. Ratio values obtained for the 3 myoglobins in this manner were compared. Because the relative heme contents were of interest, rather than the absolute contents, a pyridine hemochromogen standard curve was not prepared.

Acid-cleavage of hemes

According to Lewis (1954), who originated the method, acid cleavage data detects differences in heme-protein linkages. The procedure of Lewis (1954) was modified to permit the analysis of small amounts of protein. Mb I_A, Mb I_B and Mb II samples were dialyzed against deionized water until free of phosphate. They were then adjusted to approximately the same concentration (0.4-0.5 mg. per ml.) and NaCl was added to make the final ionic strength of each 0.02.

The desired amount of 0.04 N HCl was added to a 5 ml. beaker. The volume was adjusted to 0.50 ml. with 0.02 M NaCl solution. The beaker was then swirled while 0.80 ml. of the myoglobin solution was added. The pH was immediately measured and the contents were transferred to a 40 ml. centrifuge tube. Eight ml. of acetone were added and the tube was stoppered, swirled until cloudiness was observed throughout the contents, and centrifuged for 3 minutes at 2500 rpm. An aliquot of the supernatant was transferred to a cuvette and read spectrophotometrically at 382 mu. The method was repeated varying the proportions of 0.02 M NaCl and 0.04 N HCl, until a pH range of approximately 2 to 6 was covered. The highest absorbancy value denoted 100 percent cleavage and the lowest value zero percent cleavage. Cleavage percents were then calculated for intermediate absorbancy values, and were plotted against the corresponding pH figures. The log of the expression $(A\ pH\ 2 - A\ pH\ x)/(A\ pH\ x - A\ pH\ 6)$ was also plotted against pH. A represented the absorbancy at the indicated pH and A pH x the absorbancy at an intermediate pH value.

Because of the small amount of Mb I_B available, only a few determinations were made with this protein. The procedure was repeated at a later date for Mb II using a higher concentration of the protein.

Autoxidation studies

Autoxidation measurements were made on all 3 myoglobins simultaneously. The procedure used was essentially that of Snyder and Ayres (1961). The samples in 0:6 M phosphate buffer at pH 5.91 were placed in test tubes, which were submerged in water at the desired experimental temperature. Solid sodium dithionite was added to each solution to make the final concentration 0.04 percent. The solutions were shaken, transferred to cuvettes and read spectrophotometrically at 580 mu. The solutions were then poured back into the test tubes, shaken in air, transferred back to the cuvettes and re-read at 580 mu. This procedure was repeated until maximum absorbancy values at 580 mu were obtained. The cuvettes were then left in the cell compartment for the duration of the experiment. The automatic positioner moved the cuvettes every 15-17 seconds. Readings at 580 mu were taken at irregular intervals for a period of 2 to 5 1/2 hours depending on the temperature. The temperature was controlled for different experiments at 14.5, 19.0, and 29.5°C. to ± 0.5°C by forcing water at the desired temperature through thermospacers placed on each side of the cell compartment. After completing a run, a few crystals of potassium ferricyanide were dissolved in each cuvette and a final reading at 580 mu was taken.

The highest absorbancy value at 580 mu represented 100 percent oxymyoglobin and the value after adding the ferricyanide represented zero

percent oxymyoglobin. From these figures the percent of oxymyoglobin represented by intermediate values were then calculated. The log of percent oxymyoglobin was plotted against time and rate constants were calculated from the slopes of the resulting straight lines.

Inhibition of cytochrome c reduction

Fridovitch (1962) found that crudely-purified pig and horse heart myoglobin preparations competitively inhibited the reduction of cytochrome c by xanthine oxidase. The inhibitory properties of the separated globins were greater than those of the native myoglobins. Also, the globin and myoglobin preparations from pig muscle had greatly different inhibitory constants than those of horse heart. Thus, the inhibitory power of a myoglobin would seem to depend upon its specific globin structure. The inhibition assay could, then, indicate differences in the globin portion of the 3 beef myoglobins.

Milk xanthine oxidase (40 Worthington units) was obtained from the Worthington Biochemical Corp. and dialyzed against several changes of 0.01 M phosphate buffer at pH 6.8. It was then adsorbed onto a 2.2 by 20 cm. column of hydroxylapatite ($Ca_5 (PO_4)_3 OH$).

The hydroxylapatite was prepared by the method of Tiselius et al. (1956). One liter of 0.5 M calcium chloride and one liter of 0.5 M dibasic sodium phosphate were allowed to drip at an equal rate from 2 separatory funnels into a glass beaker while stirring. The supernatant was decanted and the precipitate was washed 4 times with deionized water. Water was again added to a volume of 2 liters and then about 50 mls. of

a freshly-prepared solution of 40 percent (by weight) NaOH were added. The mixture was boiled for one hour under constant stirring. After all but the most highly dispersed material had settled, the supernatant was decanted. The precipitate was again washed 4 times with water. Sodium phosphate buffer (0.01 M, pH 6.8) was added, and the suspension was heated until it just began to boil. After decantation, the same buffer was added and the suspension was then boiled for 5 minutes. The operation was repeated with 15 minutes of boiling in the 0.01 M buffer, and then twice more with 15 minutes of boiling in 0.001 M phosphate buffer at pH 6.8. A thick slurry of the hydroxylapatite was poured into the column and allowed to settle under gravity-flow conditions.

The xanthine oxidase was purified by chromatography according to the procedure of Fridovitch (1962). Three hundred ml. of 0.10 M potassium phosphate at pH 6.8 were added to elute any inactive protein. The phosphate concentration of the eluting buffer was then increased to 0.20 M, and elution was continued until the specific activity of the eluent reached 10.0. Specific activity was defined as units per ml. divided by the absorbancy at 280 mu. In keeping with Fridovitch's (1962) definition, a unit of xanthine oxidase was defined as the amount of enzyme which catalyzed an increase of 0.35 absorbancy units per minute at 295 mu when acting in a 3.0 ml. medium of 5×10^{-5} M xanthine, 0.05 M potassium phosphate and 1×10^{-5} M ethylenediamine tetraacetate (EDTA). The assays were performed under aerobic conditions, at pH 7.8 and at room temperature (23 ± 1°C).

At this point, 0.50 M phosphate buffer at pH 6.8 was applied to complete the elution. The active fractions were combined and brought to

75 percent saturation with solid Am SO_4. The precipitated xanthine oxidase was collected by centrifugation and stored under saturated Am SO_4 at -20°F. The enzyme was still very active after 4 months of storage. Prior to use, the Am SO_4 was removed by dialysis against 0.05 M phosphate buffer at pH 7.8.

Sigma, highly purified (type III), oxidized horse heart cytochrome c and the 3 myoglobins were also dialyzed against the 0.05 M phosphate buffer prior to experimentation.

The reduction of cytochrome c was followed at 550 mu using the automatic absorbancy recording procedure previously described. All experiments were performed at room temperature (24 ± 1°C) under aerobic conditions. Each cuvette contained 0.50 u M xanthine, 0.02 u M EDTA, 300 units (Worthington) of catalase and various amounts of myoglobin and cytochrome c in a final volume of 2.50 ml. The reaction mixture was buffered at pH 7.8 by 0.05 M potassium phosphate. The reaction was initiated by adding 0.010 ml. of xanthine oxidase (7 ug.) and stirring.

The velocity of the reaction was measured as the change in absorbancy at 1.50 minutes. The reciprocal of the cytochrome c concentration in moles per liter was plotted against the reciprocal of the velocity (v). The inhibitory constant (Ki) for each of the myoglobins was calculated from the double reciprocal plots in a manner described by Neilands and Stumpf (1958). The slope of an inhibitor-assay plot equals $\frac{Km}{V}\left(1 + \frac{(I)}{Ki}\right)$, where Km represents the Michaelis constant, V the maximum velocity and (I) the molar concentration of the inhibitor. Km and V were calculated from experimental runs in which no inhibitor was present. The slope of this plot equals $\frac{Km}{V}$ and the ordinate intercept equals $\frac{1}{V}$.

RESULTS AND DISCUSSION

Detection and Isolation of Multiple Myoglobins

Electrophoresis

Failure in this study to demonstrate heterogeneity of myoglobin by paper or moving boundary electrophoresis does not reflect upon the results of other workers (Rossi-Fanelli and Antonini, 1956; Rossi-Fanelli *et al.*, 1960; Lewis and Schweigert, 1955), all of whom detected heterogeneity by one of these methods. Neither of these electrophoretic methods were throughly investigated in this study. In the paper electrophoresis method, Rossi-Fanelli and Antonini (1956), and Rossi-Fanelli *et al.* (1960) used buffers of lower ionic strength than were employed in this study. In the moving boundary method, Lewis and Schweigert (1955) used a longer migration time than was herein employed.

Rossi-Fanelli and Antonini (1958), Rossi-Fanelli *et al.* (1960), Rumen (1959), and Akeson and Theorell (1960) used either paper or moving boundary electrophoresis to test the purity of isolated myoglobin fractions. The results of the present study suggest that starch gel electrophoresis is superior to the other electrophoretic methods for demonstrating myoglobin purity. The superior resolving ability of starch gel electrophoresis for many proteins has been well-established (Heftmann, 1961).

As previously mentioned, myoglobin samples separated into twice as many protein-staining zones on starch gels made with 7 M urea as on non-urea gels, but into the same number of heme-staining zones. Each heme-

staining zone was also a protein-staining zone. Although the 3 heme-staining zones required a longer (3-4 hours) period of time to migrate the same distance through the urea-starch gels as through the non-urea gels, they attained the same positions relative to one another in both types of gel. It was not surprising to find that myoglobin dialyzed against 7 M urea presented an absorption spectrum markedly different from that of a control sample, resembling it only in having an absorption band at the same wavelength in the Soret region. Urea would be expected to have an unfolding effect on myoglobin, i.e. by its ability to break non-covalent cross linkages (Malmstrom, 1958). However, electrophoretic results indicated that urea not only denatures myoglobin, but ruptures the globin into 2 parts. This view is inconsistent with generally accepted theories of urea-protein interaction.

The possibility of myoglobin complexing with borate ions or with metal ion contaminants during electrophoresis, or of polymer formation can be disregarded, since it was only the number of non-heme zones that differed in the 2 gel types. The use of urea in electrophoresis was discontinued at an early stage of this study. Its effect on myoglobin was not further investigated.

The relative proportions of the 3 heme-protein zones detectable by starch gel electrophoresis were not quantitatively estimated. Different crude myoglobin samples prepared by Am SO_4 fractionation alone, or by Am SO_4 fractionation combined with heat treatment, Sephadex chromatography, or crystallization, all produced the same visual results.

The use of KCN in the electrophoretic experiments, as previously described, did not noticeably affect the relative proportions or positions of the 3 heme-staining zones.

Chromatography

Am SO_4-fractionated myoglobin preparations were partially purified by Sephadex chromatography. The 280 mu/525 mu absorbancy ratio values of the collected eluent fractions decreased from high values initially (10 to 15) to values of between 3.9 and 4.5. The initial values were dependent upon the amount of colorless protein present in the preparation. The absorbancy at 525 mu was chosen as an indicator of myoglobin concentration, since it is at this wavelength that the three normal derivatives, met-, oxy-, and reduced myoglobin, have equal absorptivities. There was no clear-cut separation of proteins on the Sephadex columns, but only one broad peak. Also, the 280 mu/525 mu ratio values did not become constant, but fluctuated between 3.9 and 4.5. It was this last observation that prompted a re-appraisal of the literature on the occurrence of multiple myoglobins in various animal tissues and dictated the course of this study.

A crystalline preparation of bovine myoglobin gave a 280 mu/525 mu absorbancy ratio value of 4.41, as compared to a value of 4.24 reported by Fox (1961) for aged myoglobin crystals.

Two typical elution patterns (figures 1 and 2) from CM cellulose columns are presented. The first shows the elution pattern of a 10 ml., Sephadex purified, myoglobin sample in 0.02 M phosphate buffer at pH 6.90

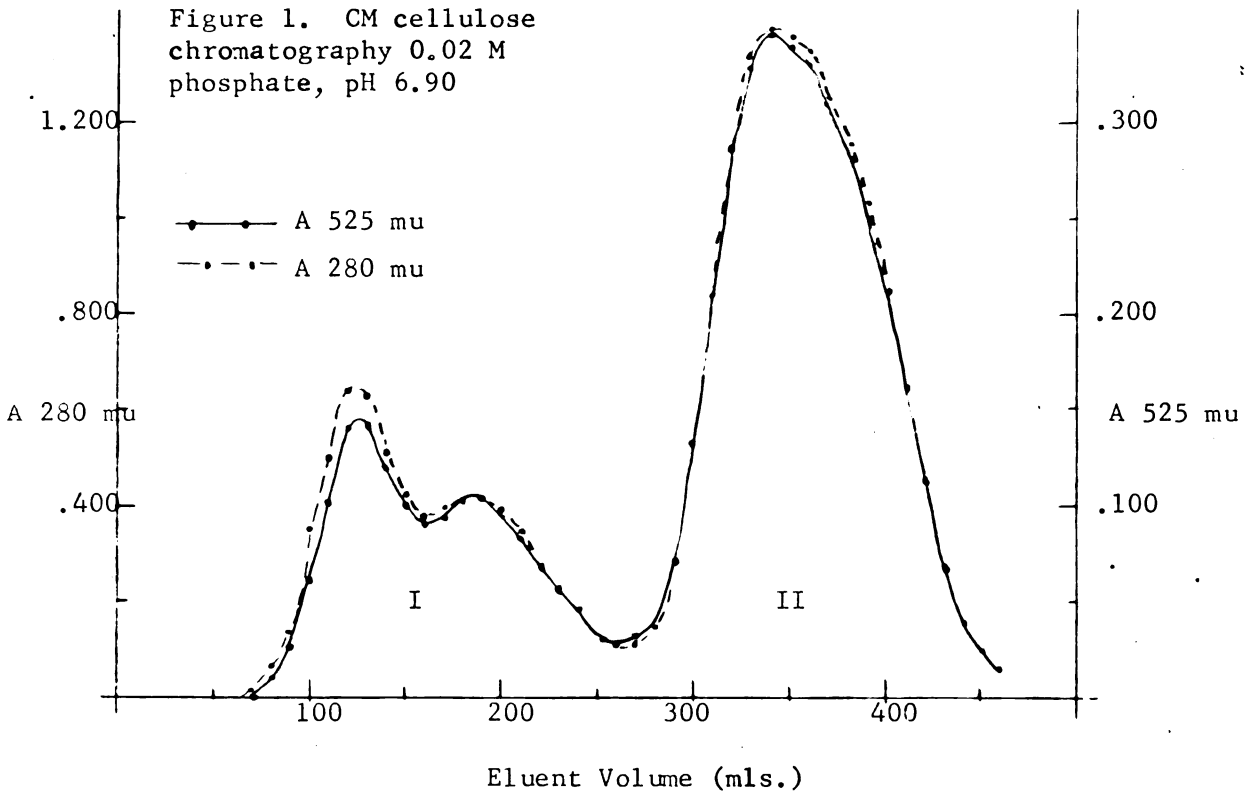


Figure 1. CM cellulose chromatography 0.02 M phosphate, pH 6.90

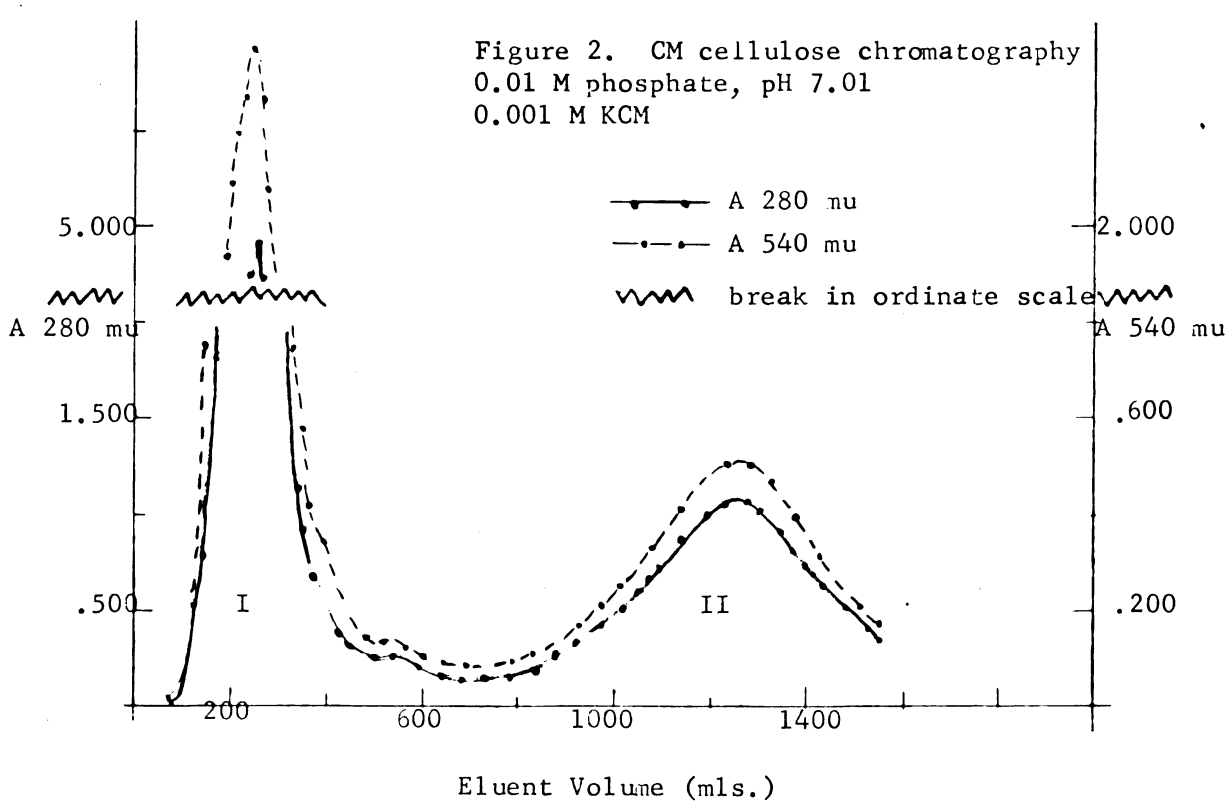


Figure 2. CM cellulose chromatography
0.01 M phosphate, pH 7.01
0.001 M KCM

from a 4.1 by 16 cm. bed of CM cellulose containing 0.45 meq. acidic groups per g. dry weight. The second (figure 2) shows the pattern of an 80 ml. myoglobin sample, which was not purified on Sephadex. The second pattern was developed in 0.01 M phosphate buffer at pH 7.01 containing 0.001 M KCN on a 4.7 by 24 cm. bed of Whatman CM cellulose.

The 2 patterns shown represent the extreme conditions employed in CM cellulose chromatography. No matter which set of conditions were employed, neither of the fractions, represented in the figures as I and II, were homogeneous. Each was a mixture of all 3 of the electrophoretically-detectable myoglobins.

However, a rough fractionation of the myoglobins was obtained on these columns. Fraction I was composed largely of the 2 fastest electrophoretically-migrating zones and fraction II of the slowest migrating zone. The CM cellulose columns also served to further eliminate colorless protein contamination. Akeson and Theorell (1960) found cytochrome c to be a contaminant of myoglobin solutions fractionated with Am SO_4, but that it remained at the top of the CM cellulose column, while the myoglobin was eluted. The formation of a stationary red band at the top of the column was also noted in this study. The band was formed regardless of whether or not the myoglobin sample had been previously purified on Sephadex. All attempts to elute this red band failed.

As previously mentioned, chromatography of fractions I and II on DEAE columns with 0.02 M tris buffers at either pH 7.83 or pH 8.00 seemingly resulted in further myoglobin fractionation. Fraction I separated

into 6 colored bands and fraction II into 4. The bands were clearly observed on the columns, yet, when tested on starch gel, they were each found to be composed of a mixture of the 3 electrophoretically-detectable myoglobins. The band that moved the fastest on the column was mostly composed of the slowest electrophoretically-migrating zone. Conversely, the band that moved the slowest on the column was mainly made up of the fastest migrating zone. The relative proportions of the 3 migrating zones in the other bands varied according to the relative position of the band on the column.

Noticeably different results were obtained when fractions I and II were treated with KCN prior to chromatography. Only 3 distinct bands were formed. A 4th band was observed following closely behind the fastest moving band, but was very faint. The 3 major bands were tested electroporetically and again were shown to contain all 3 of the starch gel-detectable myoglobins. The observation that fewer bands were formed on KCN treatment agrees with the findings of Perkoff et al. (1962). These authors concluded that myoglobins, differing only in the state of the iron in their heme prosthetic groups, i.e. acid and alkaline metmyoglobins, could be chromatographically resolved.

Since 4 bands formed on chromatographing the cyanide derivatives of the myoglobin fractions, there should have been 8 bands on chromatographing the metmyoglobins, i.e. 4 acid and 4 alkaline metmyoglobin bands. As previously mentioned, a maximum of only 6 bands were observed. However, the other bands could easily have been obscured. Indeed, it is probable, since both fractions I and II contained all 3 of the electrophoretically-

detectable myoglobins, and yet, on chromatographing the met- forms, fraction I separated into 6 bands and II into only 4 discernable bands.

DEAE cellulose chromatography, using the same buffer throughout complete development, roughly fractionated the myoglobins. Thus, the techniques used for horse myoglobin separations (Akeson and Theorell, 1960) were, at least, roughly applicable to the separation of multiple beef myoglobins. The 3 chromatographic fractions were labelled I_A, I_B and II and were used for characterization analyses during the early stages of this study. Fractions I_A, I_B and II corresponded to the horse myoglobin fractions II_1, II_{11} and I, respectively, isolated by Akeson and Theorell (1960).

Since each of the 3 fractions contained substantial amounts of the other 2 electrophoretically-detectable myoglobins, chromatographic experimentation was continued.

Various stepwise development techniques were tried, but did not result in better separations. The stepwise technique, which was ultimately adopted, seemed to involve a displacement effect rather than to be a ture elution process. Mb II was eluted from the column in a broad, diffuse band by the 0.02 M tris buffer. Immediately upon addition of the 4:1 tris-phosphate buffer, Mb I_A began to move rapidly down the column in a narrow dark band. Very little tailing was noted. Mb I_B exhibited the same behavior when phosphate buffer alone was added in the next step. The sharper resolution obtained by this method does not appear to be due to pH or ionic strength effects, since the previously-tested stepwise methods, in which tris buffers of successively lower pH values or of

successively higher ionic strengths were applied to the columns, did not improve the fractionation.

The electrophoretic patterns of various myoglobin fractions are exhibited in the accompanying photographs (figures 3A and 3B). The protein migrated from the gel slots, seen at the right of the photograph, to the positions indicated by the stained zones at the left. Figure 3A demonstrates the 2 staining techniques used in this study. Patterns 1 to 6 were all formed on the same gel. The gel was divided in half prior to staining. The top half, containing patterns 1, 2 and 3, was stained with the protein dye. The other half, containing patterns 4, 5 and 6, was stained by the heme-detecting method previously described. Examination of the photograph illustrates that the protein-staining method caused gel shrinkage and left a dark background. Patterns 1, 2, 4 and 5 were formed from identical samples of the same crude, Am SO_4-purified myoglobin preparation. Patterns 3 and 6 were formed from duplicate samples of the main fraction (II) obtained on CM cellulose chromatography of the crude myoglobin preparation. Comparing the 2 gel halves, it is evident that the heme-staining method is much more sensitive for the detection of myoglobins.

Figure 3B pictures myoglobin fractions at various stages of chromatographic separation. All 3B patterns were stained by the heme-detecting method. Patterns 8 and 11 were both formed from the main fraction, II, obtained on CM cellulose chromatography. Pattern 12 represents the smaller fraction, I, obtained on CM cellulose chromatography. A rough fractionation of crude myoglobin on the CM cellulose columns is revealed by comparing patterns 11 and 12.

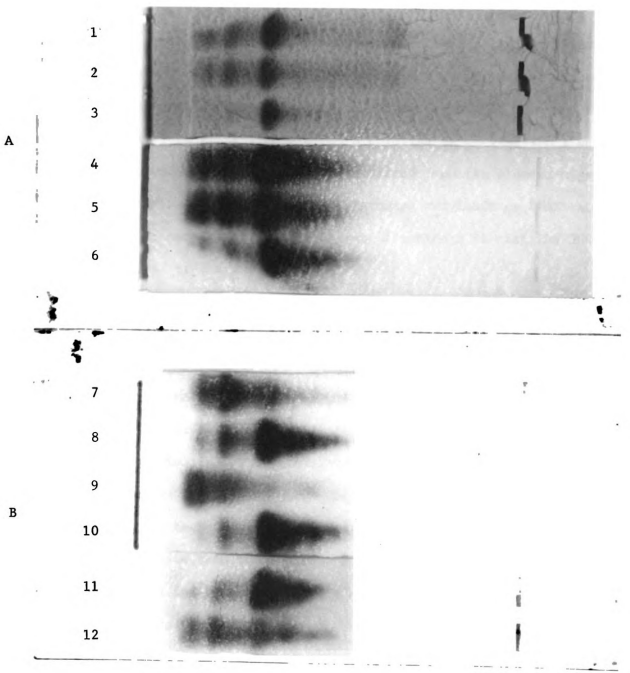


Figure 3. Starch gel electrophoretic patterns.

A. Demonstration of difference between the 2 staining methods used.

B. Patterns of myoglobin fractions at various stages of chromatographic purification.

Patterns 7, 9 and 10 represent the fractions Mb I_A, Mb I_B and Mb II, respectively. These fractions were obtained on DEAE cellulose chromatography using the tris-phosphate, stepwise procedure ultimately adopted. Comparing pattern 12 with patterns 7 and 9, it is evident that the 2 fastest electrophoretically-migrating myoglobins in CM fraction I were largely resolved on DEAE columns and separated from the slowest migrating zone. A purification of the slowest migrating myoglobin on DEAE cellulose is revealed by comparing the CM fraction II (pattern 8) with the DEAE fraction, Mb II (pattern 10).

The fractions, Mb I_A, Mb I_B and Mb II, are each shown to be heterogeneous by starch gel electrophoresis. However, comparison of patterns 1 and 2 with patterns 4 and 5 in figure 3A indicates that the heme-staining method over estimates the relative proportion of minor components in a pattern. Thus, it is believed that the heterogeneity of the myoglobin fractions used in final characterization studies is not as prominent as indicated to the naked eye. The myoglobin fractions obtained by the chromatographic techniques of Akeson and Theorell (1960) appeared much more heterogeneous than those pictured herein.

Pattern 12 indicates that Mb II, represented electrophoretically as the heavy, slowest migrating zone in patterns 1, 2, 3, 4, 5, 6, 8, 10 and 11, is composed of at least 2 distinct myoglobins. The minor, tailing zone probably corresponds to the 4th faint band previously mentioned as closely following fraction II on DEAE chromatography. Thus, there would appear to be at least 4 myoglobins occurring in bovine muscle.

It is estimated from observations on both electrophoretic and chromatographic analyses that the relative proportions of the 3 myoglobin fractions, Mb I_A, Mb I_B and Mb II occur in the percentages 10-15, 2-5 and 80-88, respectively.

Characterization Studies

Absorption spectra

The absorption spectra for the met-derivatives of Mb I_A, Mb I_B and Mb II are presented in figure 4. The spectra of the carboxy-derivatives are compared in figure 5. Both figures show that the 3 myoglobins absorb at the same wavelengths, but that they differ in absorptivities at most wavelengths. Rumen (1959) reported that the 5 myoglobins he isolated from seal muscle also differed in absorptivity values but not in positions of maxima and minima. Conversely, Akeson and Theorell (1960) reported that the 3 myoglobin fractions which they isolated from horse muscle had identical absorption spectra. It is interesting that the 3 myoglobin fractions prepared earlier in this laboratory by the chromatographic techniques described by Akeson and Theorell (1960) also had almost identical absorption spectra. Thus, spectral results augment electrophoretic results in confirming the crudeness of the separation techniques of Akeson and Theorell (1960), at least for beef myoglobins.

In preparing the spectral curves of figures 4 and 5 a molecular weight of 17,000 was assumed for each of the 3 myoglobins. The absorbancies of Mb I_A, Mb I_B and Mb II at 215 mu divided by their absorbancies at 225 mu gave ratio values of 1.47, 1.47 and 1.48, respectively. The con-

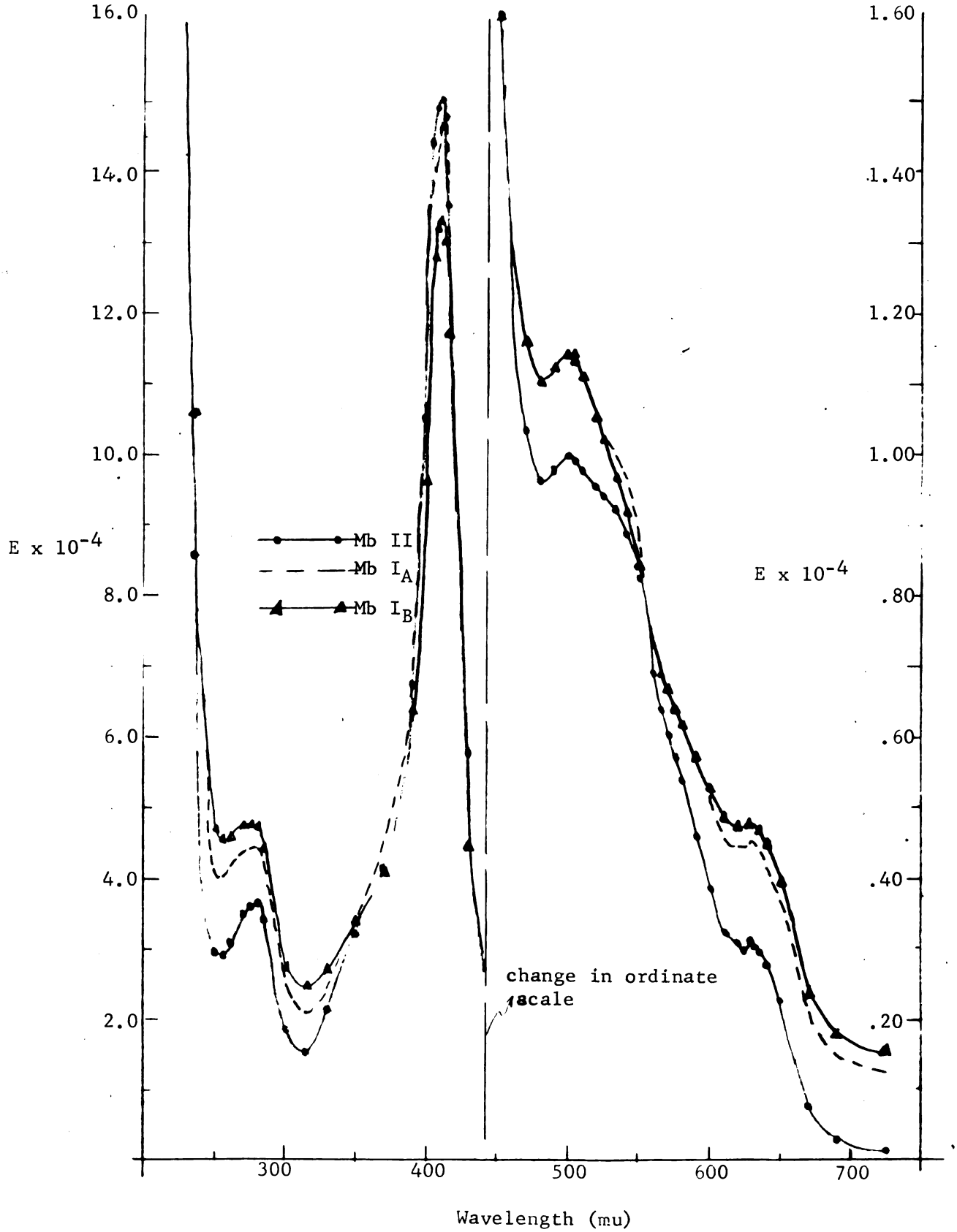


Figure 4. Metmyoglobin absorption spectra pH 6.90

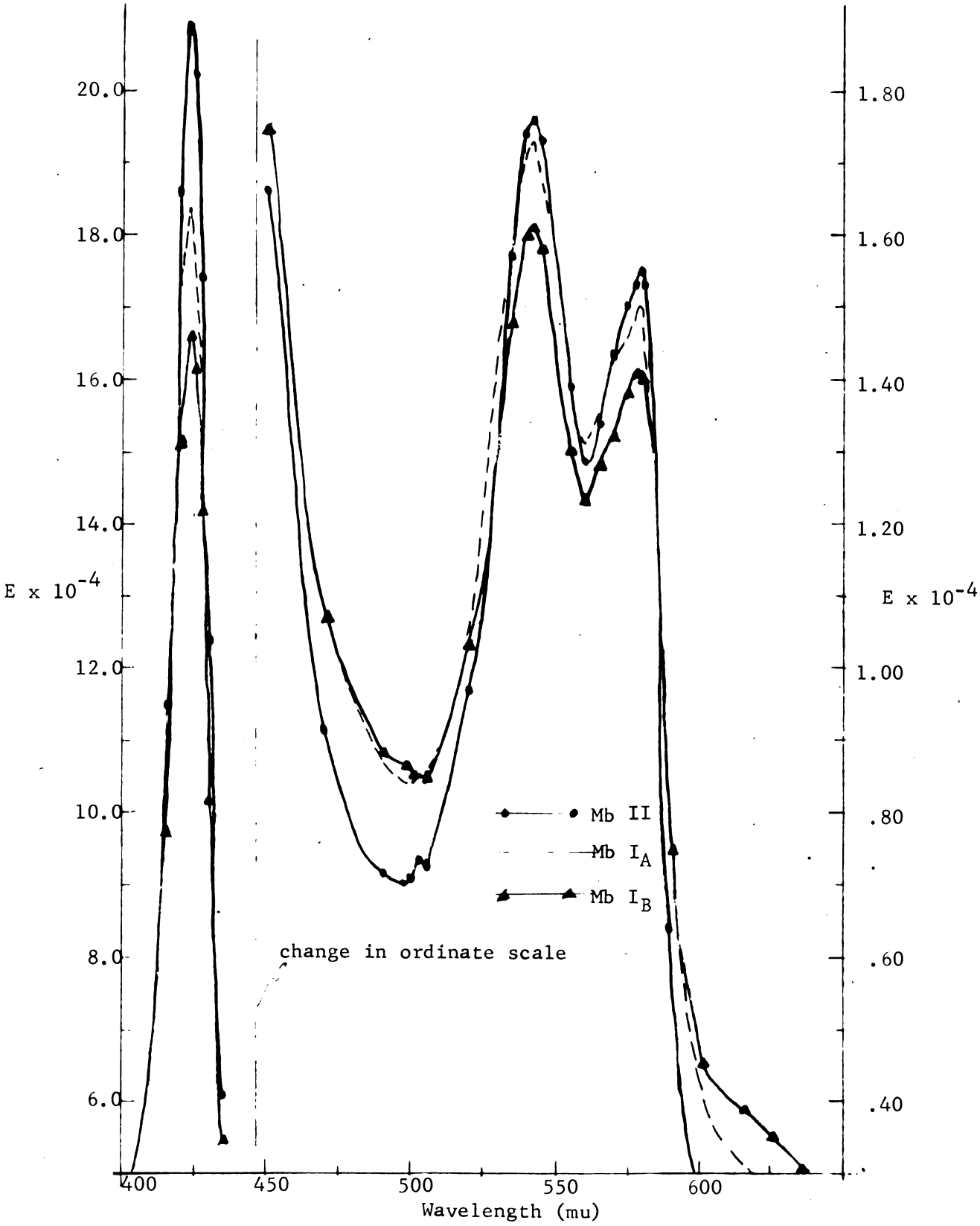


Figure 5. Carboxy myoglobin absorption spectra

stancy of the ratio values suggest either that the method used to determine protein content (Waddell, 1956) successfully eliminated the effect of specific amino acid absorption at these wavelengths, or that the 3 myoglobins contain equal amounts of the amino acids absorbing at these wavelengths.

The absorption maxima for the met-derivatives occur at wavelengths of 628, 502, 410 and 278 mu. These wavelengths agree to within 2 mu with values reported for metmyoglobin preparations from the horse (Bowen, 1949), whale (Smith and Gibson, 1959) and seal (Rumen, 1959). The absorption maxima of the met-derivatives of Mb I_A, Mb I_B and Mb II, however, occur at wavelengths that suggest the partial formations of the alkaline form of metmyoglobin. Since Bowen's (1949) spectrum was taken in pH 7.0 buffer and the spectra in this study were determined in pH 6.90 buffer, the pK of the reaction (acid Met Mb $\rightleftharpoons$ alkaline Met Mb) must be lower for beef myoglobin than that reported for horse myoglobin (George and Hanania, 1952). The spectra of metmyoglobins I_A, I_B and II, when taken in pH 6.50 buffer, exhibited the same wavelengths of minima absorption (590, 465 mu) as reported for other species (Bowen, 1949; Rumen, 1959).

Absorption maxima and minima of the carboxy-derivatives of Mb I_A, Mb I_B and Mb II occur at the same wavelengths as reported for other species (Bowen, 1949; Smith and Gibson, 1959; Rumen, 1959). The molar absorptivities of the 3 myoglobins at positions of maxima and minima are listed and compared with literature values in table 1.

From table 1, it is obvious that spectral curves prepared by different authors can be compared only as to general shape. The absorbancy

Table 1. Millimolar absorptivity values for the carboxy-derivatives of various myoglobins

Wavelength in mu	horse[1]	whale[2]	seal[3]	Mb I_A	Mb I_B	Mb II
578	12.9	15.1	12.8	15.1	14.1	15.5
560	10.6	--	10.5	13.1	12.3	12.8
542	14.8	17.0	14.5	17.3	16.1	17.6
502	5.3	--	5.0	8.5	8.5	7.3
423	--	187	206	184	166	209

[1]from Bowen (1949). crystalline myoglobin
[2]from Smith and Gibson (1959)
[3]from Rumen (1959). myoglobin I from chromatographic separation.

ratio values, at different wavelengths in the 500-600 mu range, of Mb II and Rumen's (1959) myoglobin I are very similar. Rumen's (1959) myoglobin I composed 90 percent of the total seal myoglobin chromatographed and had a molecular weight of 18,600.

Heme content

The relative heme contents of the myoglobins, I_A, I_B and II, indicated that all 3 had the same molecular weight. The absorbancy reading of the pyridine hemochromage at 557 mu was divided by the protein content as previously described. The ratio values obtained were 1.48, 1.48 and 1.50 for Mb I_A, Mb I_B and Mb II, respectively. The absolute molecular weights of the 3 myoglobins could have been calculated from the absorbancies at 557 mu if the absorptivity of pyridine demochromogen had been known. As mentioned previously, absorptivity measurements were not made in this study. The pyridine hemochromogen absorptivity value could not be extracted from the literature as several conflicting values have been reported (Lemberg and Legge, 1949).

Heme cleavage

The results of acid cleavage experimentation are presented in figures 6 and 7. The curves of figure 6 demonstrate the susceptibilities of the 3 myoglobins to acid cleavage. Figure 7 presents the same data used for the figure 6 plots, but in a logarithmic expression. Mb I was plotted at only a few points because of the small quantity of this protein available. Examination of figures 6 and 7 reveals that there are 2 curves presented for Mb II. The curves of Mb I_A, Mb I_B and Mb II were made using roughly the same protein concentrations. The second curve of Mb II, labelled Mb II*, was made using this protein at a threefold higher concentration.

As illustrated by figures 6 and 7, the plots labelled Mb II and Mb II* are distinctly different. Since the same myoglobin sample was used in plotting both Mb II and Mb II* curves and differed only in concentration, the validity of the method was questioned. The results should have been independent of concentration since they were based on percentages.

Lewis (1954), who originated the method, and Lewis and Schweigert (1955) determined the percent of heme cleavage at various pHs for crystalline beef myoglobin. In the second paper, the authors (1955) obtained distinctly different results than those presented in the first paper (Lewis, 1954), but apparently ignored the fact. The heme cleavage plots of Mb II* in figures 6 and 7 are very similar to those presented by Lewis (1954), whereas those of Mb II are distinctly different.

It was noted in this study that at high absorbancy readings, i.e. readings greater than 1.00, the absorbancy rapidly increased during its

Figure 6. Acid cleavage of heme from myoglobins

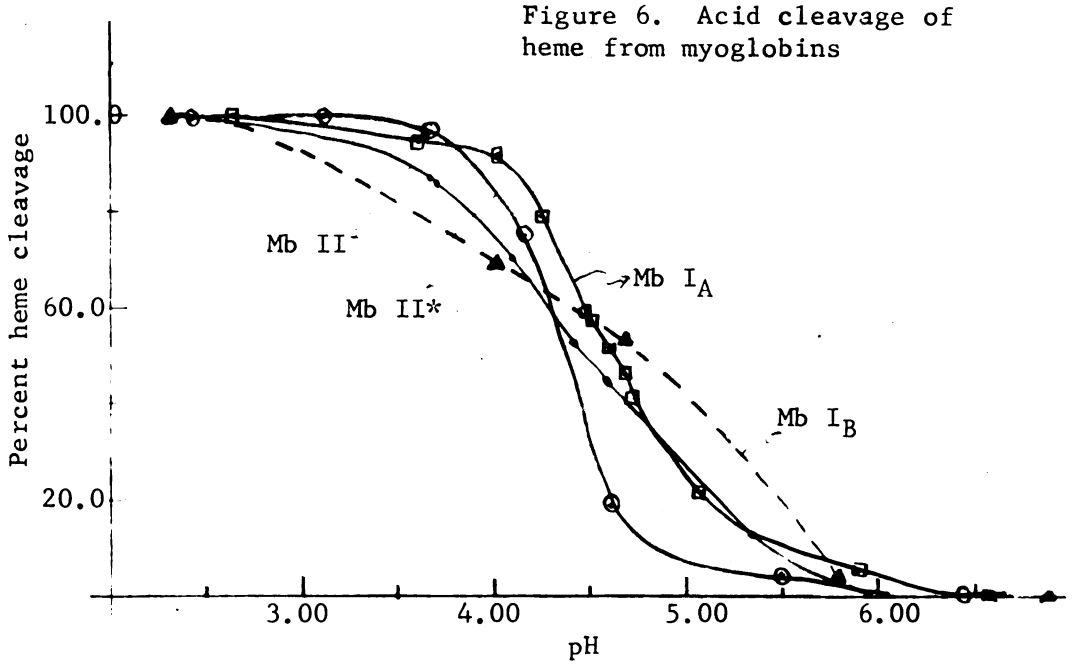
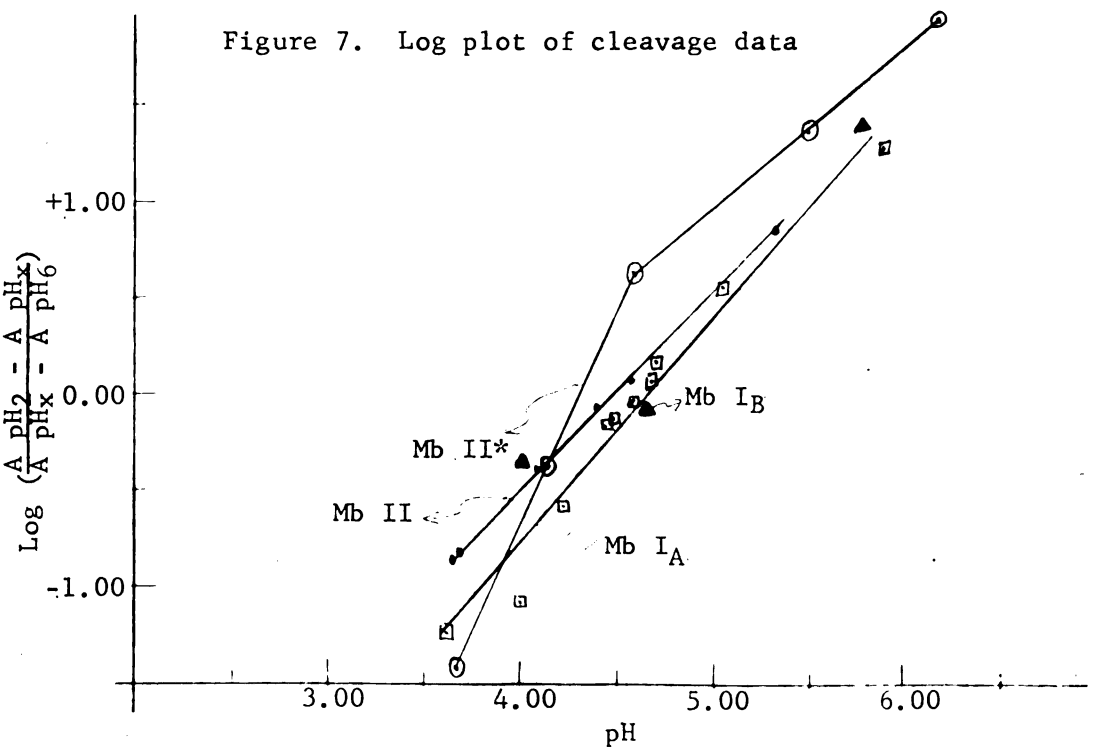


Figure 7. Log plot of cleavage data



determination. The absorbancy of the same sample did not increase significantly if it was kept in the dark. This observation suggests that hemin, concentrated in acetone, may undergo a light-catalyzed reaction involving polymerization.

Autoxidation

The autoxidation rate constants (k) for the 3 myoglobins were almost identical. The rate constants for Mb I_A, Mb I_B and Mb II at 29.5°C were 0.47, 0.47 and 0.46 per hour, respectively. At 19°C, the k values for all 3 myoglobins were 0.05 per hour. Whereas the rate constants at 19°C agreed with the literature (Snyder and Ayres, 1961; Brown and Dolev, 1963a), the k values found for the 29.5°C were all substantially greater.

Autoxidation rates were determined at various times throughout this study. It was noted that as the purity, or homogeneity, of the samples increased, the autoxidation rates at higher temperatures (29-33°C) also increased. Brown and Dolev (1963a) reported that crude myoglobin preparations autoxidized at a substantially slower rate than myoglobin purified by DEAE cellulose chromatography. They attributed this observation to a protective effect exerted by contaminating, and possibly sulfhydrl-containing, proteins in the preparation. The observation of Brown and Dolev (1963a), however, would not be applicable to the findings of this study. All the myoglobin fractions studied were considered to be free of other proteins, and only to vary in degree of freedom from the other myoglobins.

Also at the higher temperatures (29-33°C) employed in this study, it was noted that the greater the homogeneity of the samples, the more

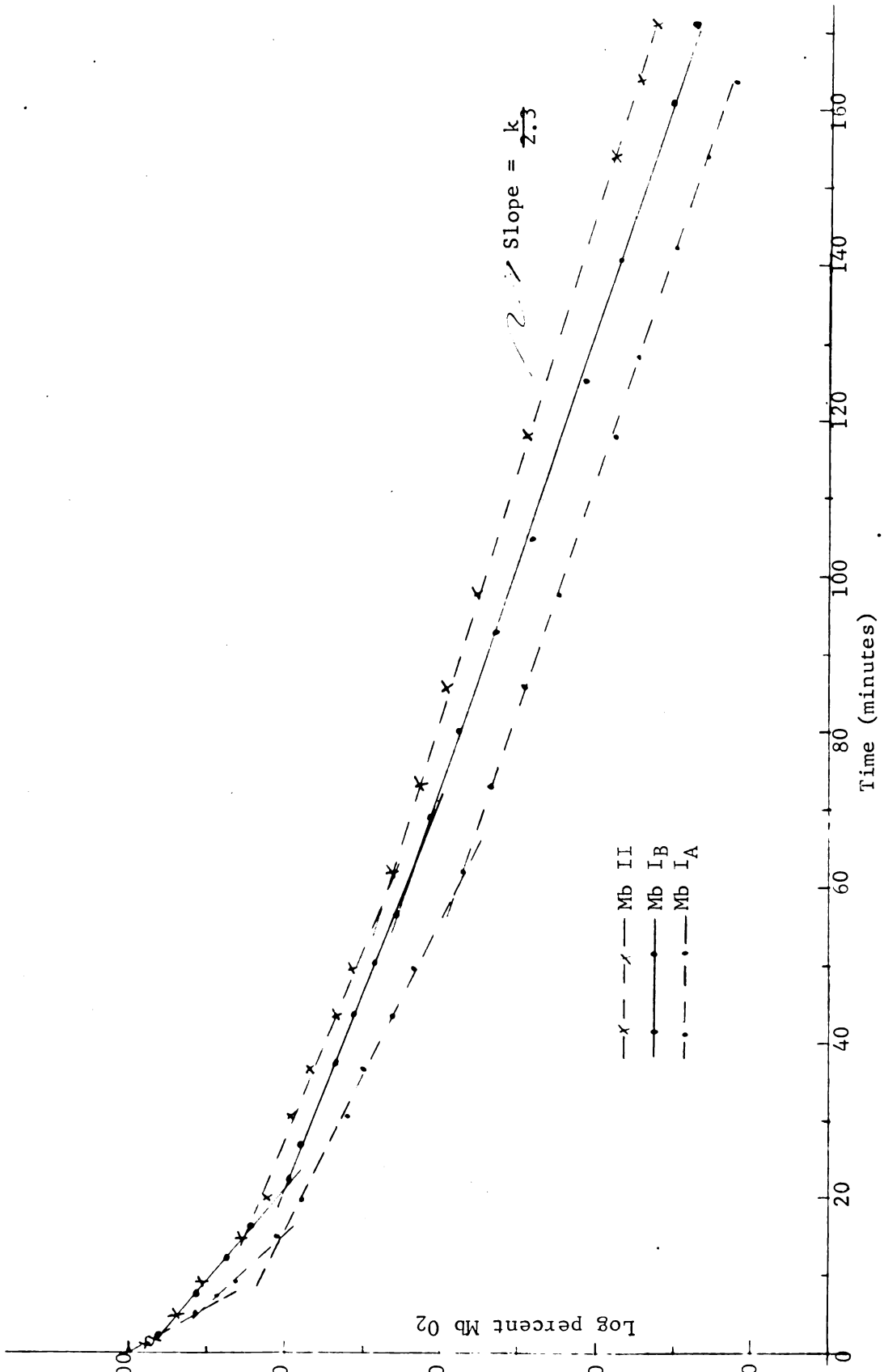


Figure 8. Autoxidation of myoglobins at 29.5°C.

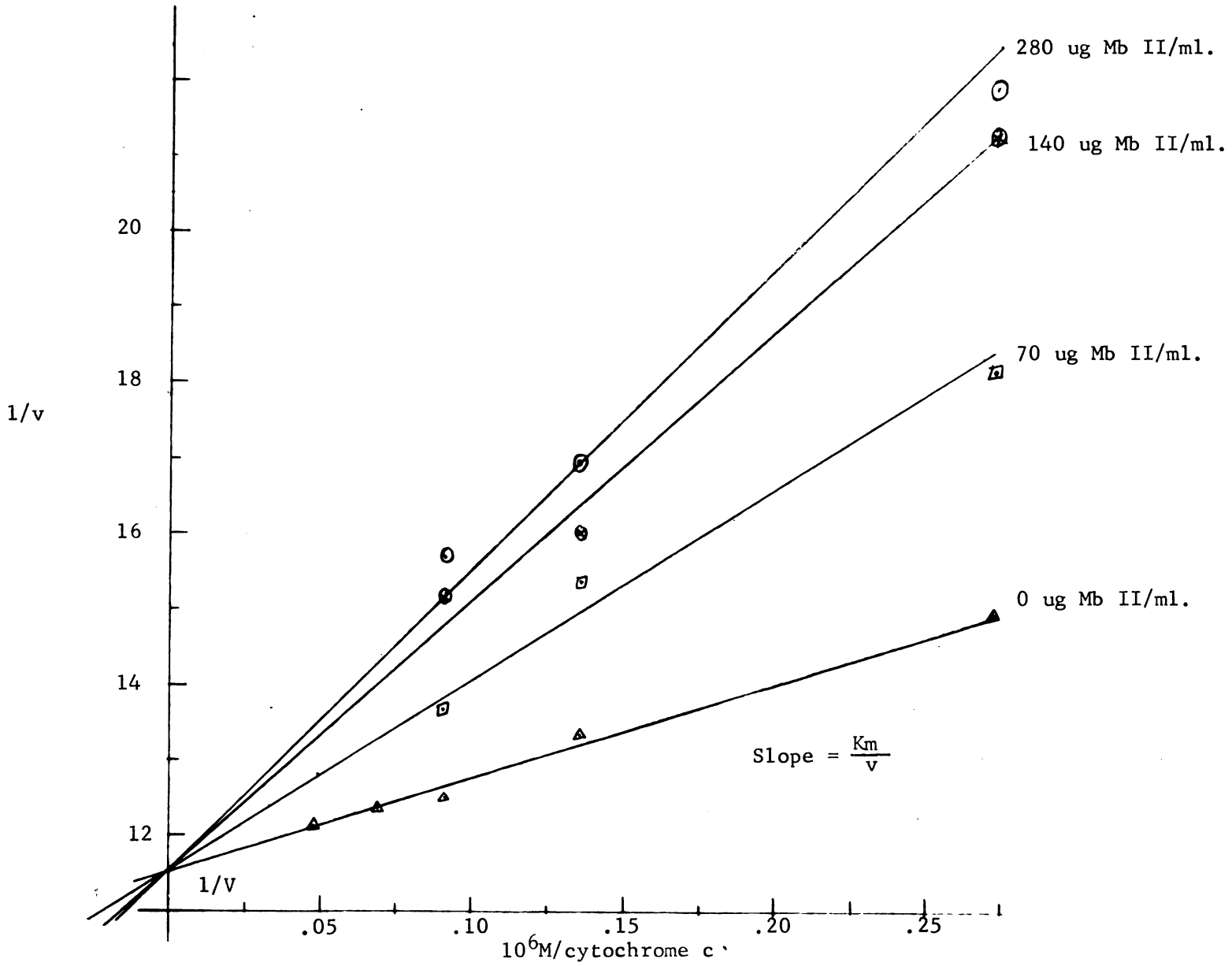
pronounced were anomalies in the initial rates of autoxidation. The amount (0.04 percent) of dithionite used in the experiments was less than the level reported by Snyder and Ayres (1961) as causing an effect on the rate constant or initial readings. Figure 8 illustrates typical autoxidation rate plots of Mb I_A, Mb I_B and Mb II at a high temperature.

Although no measurements on the rate of oxygenation of the myoglobins were attempted, it appeared that Mb I_B was oxygenated much more rapidly than the other 2 fractions.

As was pointed out in the review of literature, very little is known about the mechanisms of myoglobin autoxidation. Thus, the significance of the observations presented cannot be explained.

Inhibition of cytochrome c reduction

All 3 myoglobins were found to competitively inhibit the reduction of cytochrome c by xanthine oxidase. Figures 9, 10 and 11 indicate the extent to which Mb II, Mb I_A and Mb I_B, respectively, inhibit the enzymic reaction. The inhibitory constants of each of the 3 myoglobins were determined at 3 different concentrations, as is illustrated in figures 9, 10 and 11. The different myoglobin concentrations gave the inhibitory constant values for Mb I_A of 4.1, 4.1 and 4.5 x 10^{-7}M, for Mb I_B of 3.7, 1.7 and 1.7 x 10^{-7}M and for Mb II of 78, 46 and 68 x 10^{-7}M. It can be seen that the agreement of values for any one of the myoglobins was not exceptionally good. A longer reaction time should have been allowed. However, the differences between the 3 myoglobins are sufficiently great to allow comparisons.


280 ug Mb II/ml.
140 ug Mb II/ml.
70 ug Mb II/ml.
0 ug Mb II/ml.
1/v
20
18
16
14
12
Slope = Km/V
1/V
.05
.10
.15
.20
.25
10^6M/cytochrome c

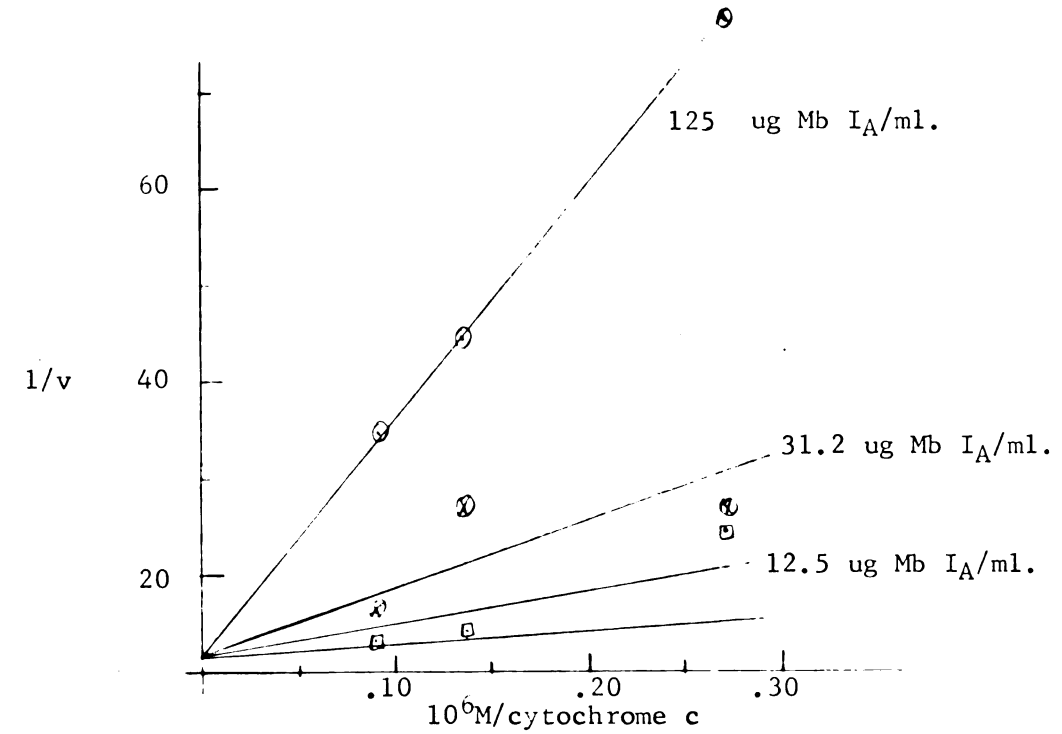


Figure 10. Mb I_A inhibition of cytochrome c reduction.

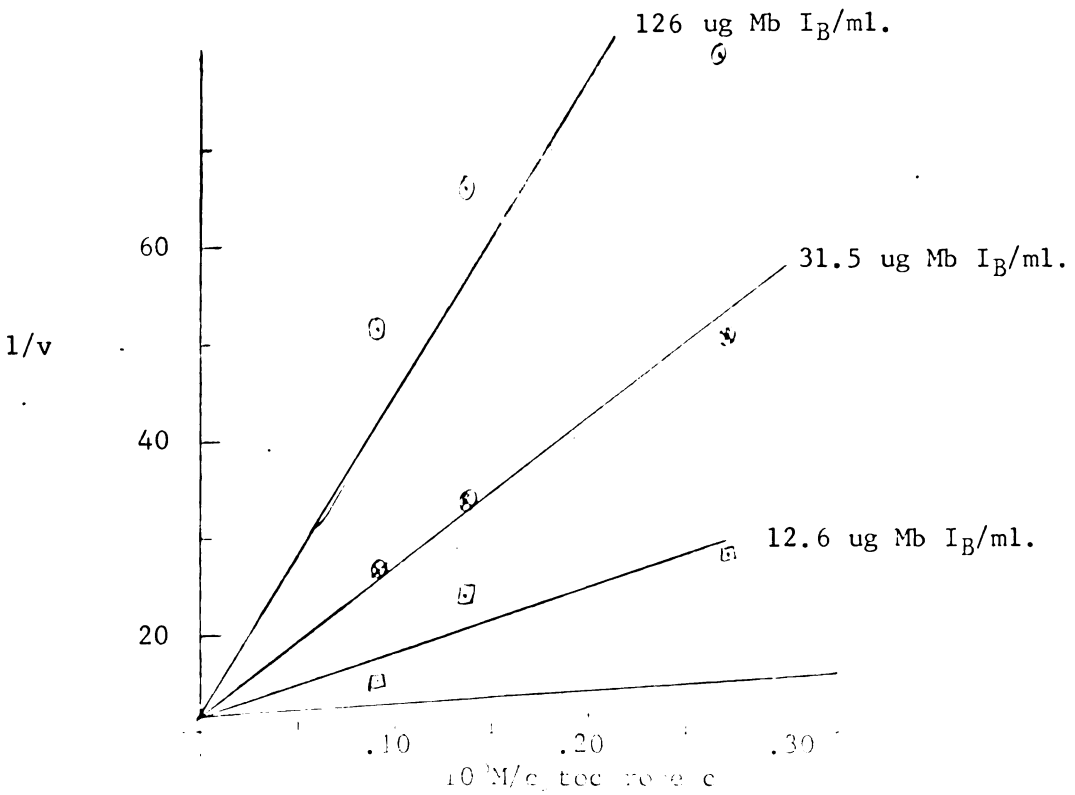


Figure 11. Mb I_B inhibition of cytochrome c reduction.

Mb I_B had, in all cases, a greater inhibitory power than Mb I_A. However, since electrophoretic analysis indicated Mb I_A to be slightly contaminated with fraction Mb II, the difference in inhibitory power between the pure proteins, Mb I_A and Mb I_B, may be less than is shown. Both myoglobin fractions I_A and I_B inhibited the cytochrome c reaction to a much greater extent (18-35 times) than did Mb II. It is interesting to note that Mb II, which makes up 80-88 percent of the total myoglobin in beef muscle, possesses much less inhibitory power than the other myoglobins.

Fridovitch (1962) found pig heart myoglobin to have an inhibitory constant of 6×10^{-7} M and a horse heart preparation to have a constant of 3.5×10^{-8}M. He determined the Km of cytochrome c to be 1.3×10^{-6}M, while the Km found for cytochrome c in this study was 1.1×10^{-6}M.

The role of myoglobin in respiratory physiology has been relegated to one of only indirect involvement; that of carrying and storing oxygen. Its interaction with the main respiratory chain is purely physical. The respiratory chain lowers the oxygen tension. Myoglobin restores it. Yet it seems odd that myoglobin and cytochrome c can have so many properties in common and not be functionally related in vivo. Both proteins are water-soluble and can be separated only with a great deal of difficulty by Am SO_4-fractionation. Both proteins contain iron-porphyrin groups and are of roughly the same molecular weight. According to West and Todd (1957), cytochrome c has a redox potential of +0.27 volts at pH 7 and cytochrome b has a potential of -0.04 volts at pH 7.4. The myoglobin redox potential is +0.046 volts at pH 7.0 (Taylor and Morgan, 1942)

and can thus be oxidized by cytochrome c and reduced by cytochrome b. The extent to which myoglobin is oxidized and/or reduced by the cytochromes is, however, a question not of thermodynamic feasibility, but of the relative rates of electron-competing, enzyme-catalyzed reactions.

Myoglobin cannot be postulated to interact directly with the cytochrome chain on the basis of available evidence. Myoglobin, itself, does not accept the electrons that it competitively inhibits cytochrome c from accepting. The fact that myoglobin does inhibit the reaction, however, presents one more, seemingly unrelated, coincidence between these 2 proteins.

The multiple myoglobins

The possibility that the 3 myoglobins, isolated and characterized in this study, are artifacts must be considered. The fact, mentioned previously, that the 3 myoglobins invariably occur in the same proportions, independent of the method of preparation, together with the characterization results suggest that the myoglobins are naturally-occurring. The similarity of autoxidation rates among the 3 myoglobins suggests that one or two of them are not denatured, as various authors (Watts, 1954) have found a relationship between denaturation and rate of autoxidation. The myoglobins are separated on chromatographic columns in the order expected from electrophoretic analysis. Also, the myoglobins exhibit the same electrophoretic behavior before and after chromatography. Thus, if the myoglobins are denatured during chromatography, they would also have to be denatured in the same way during electrophoresis.

As previously described, myoglobin is not noticeably affected by heat treatment (55°C for 5 minutes). Various authors (Gibson and Smith, 1959; Rumen, 1959; Akeson and Theorell, 1960) have noted that myoglobin can be split by acid acetone treatment into heme and globin portions, which can then be recombined to yield products having properties identical to native myoglobin. Either of these treatments would seem to be more drastic than the relatively mild conditions encountered during the preparation of Mb I_A, Mb I_B and Mb II.

Thus, the 3 myoglobins isolated from bovine muscle in this study are considered to occur naturally and not to be artifacts of the preparation techniques.

The myoglobins I_A, I_B and II differed in spectral absorptivity values, ease of heme-globin cleavage and degree of inhibiting enzymic cytochrome c reduction as well as in chromatographic and electrophoretic properties. If the heme cleavage results for the 3 myoglobins at similar concentrations are valid, then the heme-globin linkages between the myoglobins would appear to be different. The spectral results would seem to confirm a difference in the heme-globin linkages. The replacement of a single amino acid in the main portion of the globin by another amino acid, as is the difference between normal and sickle-cell hemoglobin (Ingram, 1957), or the slight rearrangement of a segment of the polypeptide chain as was found for horse myoglobins (Akeson and Theorell, 1960), would not alone account for the spectral differences found in this study. The spectral differences found were not great, implying that the main heme-globin linkages, i.e. the bonds from the globin to the iron, were

similar. The obvious possibility, then, is that the weaker bonds from the globin to the heme side chains, i.e. propionic acid groups, vary among the 3 myoglobins.

The difference among the 3 myoglobins in competing with cytochrome c for the binding site of xanthine oxidase reflects a difference in globin structure among them. Fridovitch (1962) noted that native myoglobin and globin split from myoglobin by acid acetone treatment had slightly different inhibitory powers. This observation is explained if it is assumed that the amino acids of the globin which combine with the enzyme's active site are located in close proximity to the heme. The bonding of one or more of these amino acids to the heme in native myoglobin is then possible. Thus, the inhibition results of this study can be related to spectral and heme cleavage results to postulate a difference in the heme-globin linkage among the 3 bovine myoglobins isolated.

The significance of the multiple occurrence of myoglobin cannot be evaluated until much more is known about the properties of the individual myoglobins.

SUMMARY AND CONCLUSIONS

Multiple-occurring bovine muscle myoglobins were detected by starch gel electrophoresis. The presence of at least 4 electrophoretically-distinct myoglobins was indicated. The 3 major myoglobins, labelled Mb I_A, Mb I_B and Mb II and comprising roughly 10-15, 2-5 and 80-88 percent of the total myoglobin, respectively, were separated and isolated in fairly homogeneous form by DEAE cellulose chromatography. The ultimately-adopted chromatographic procedure involved a stepwise development technique utilizing both tris and phosphate buffers.

The 3 myoglobins were found to be similar in light absorption minima and maxima wavelength positions for both their carboxy- and met-derivatives, in heme contents and in autoxidation rates. The autoxidation rates for all 3 myoglobins at high temperatures (29-33°C) were found to be higher than literature values and than values found in this laboratory for less homogeneous samples.

The 3 myoglobins were found to be dissimilar in spectral absorptivity values for both their carboxy- and met-derivatives, in ease of heme cleavage by acid and in degree of inhibiting the enzymic reduction of cytochrome c. The dissimilarities between the 3 myoglobins were postulated to be due to differences in their heme-globin linkages.

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