1968

A study of changes in bovine myoglobin fractions from carboxymethyl cellulose columns

Huda S. Felland
Iowa State University

68-10,460

FELLAND, Huda S., 1929-

A STUDY OF CHANGES IN BOVINE MYOGLOBIN FRACTIONS FROM CARBOXYMETHYL CELLULOSE COLUMNS.

Iowa State University, Ph. D., 1968
Biochemistry

University Microfilms, Inc., Ann Arbor, Michigan

A STUDY OF CHANGES IN BOVINE MYOGLOBIN FRACTIONS FROM CARBOXYMETHYL CELLULOSE COLUMNS

by

Huda S. Felland

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Food Technology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1968

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

INTRODUCTION

Proteins play a prominent role in all biological systems. Almost all chemical reactions that take place in living cells are catalyzed by enzymes and all known enzymes are proteins. For these reasons the study of proteins has attracted scientists from a wide range of disciplines. Hemoglobin and myoglobin, in particular, have been used as model systems for the study of proteins and enzymes in general. The relative simplicity of the myoglobin molecule made it the protein of choice for x-ray crystallographic studies. Elucidation of its three-dimensional structure has made it uniquely suitable for interpretation of results on the molecular level.

Investigators in medical disciplines are interested in the possible involvement of myoglobin in muscular diseases. Physiologists are concerned with its role as an oxygen carrier. Food scientists are interested in the phenomenon of autoxidation as it relates to meat color. Biochemists have been interested in all the above aspects as well as the microheterogeneity of the protein. Biological reasons for the microheterogeneity of many enzymes catalizing metabolic processes are well established. However, there seems to be no obvious biological reason for the existence of more than one type of myoglobin. Colvin et al. (1954) suggested that microheterogeneity is so commonly encountered in proteins that biochemists might well regard any given protein as a family or group of molecules having the same biological function but differing slightly in composition. In reviewing the literature on the microheterogeneity of proteins one might well subscribe to this idea.

However, a review of the literature on the microheterogeneity of myoglobin brings forth such a conglomeration of varied results that one cannot help but speculate on the artifactual nature of some of the findings.

Because a non-heme protein consistently accompanied myoglobin isolated from most species by a variety of methods, it seemed likely that the microheterogeneity of this protein might be an artifact resulting in some way from the presence of this colorless contaminant. Evidence accumulated in the course of this study pointed to the contrary. However, it soon became apparent that the number of fractions obtained in chromatography and electrophoresis of myoglobin from a single species could be varied greatly depending on the experimental conditions employed.

Consequently, this study was directed toward determining the experimental conditions which produce such widely differing results with myoglobin from a single species. Particular attention was given to column length, amounts of myoglobin chromatographed and equilibrating pH. A common denominator was sought to help explain the chromatographic and electrophoretic results of microheterogeneity that have been reported for this protein.

REVIEW OF LITERATURE

Chemistry of Myoglobin

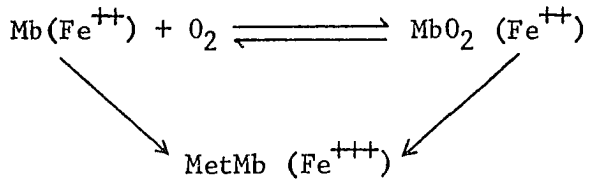
Generally vertebrate and invertebrate muscle cells differ from other cells in that their sarcoplasmic fraction contains myoglobin. Muscles of diving mammals such as the whale are particularly high in myoglobin content. This probably facilitates submersion for long periods.

Myoglobin is a globular heme protein that serves to store molecular oxygen. It accepts oxygen from the lungs via hemoglobin. In muscles at rest myoglobin is oxygenated. During contraction the demand for oxygen is greatest; as the intracellular oxygen pressure falls, oxygen dissociates from myoglobin and is released to cytochrome oxidase. Cytochrome oxidase has a greater affinity for oxygen than myoglobin, and myoglobin has a greater affinity for oxygen than hemoglobin. Much of the myoglobin is free in solution in the muscle fibers. However, Criddle et al. (1961) suggested that, in vivo, some myoglobin seems to be bound to the lipid layer on the outside membrane of the mitochondrion. This places myoglobin near the end of the electron transport system close to cytochrome oxidase. Thus the stored oxygen is readily available for reduction to water whenever the metabolic activity of the cell demands it.

In order to bind oxygen the iron in the heme has to be in the reduced or divalent state. In this form the heme-protein is referred to as reduced or deoxymyoglobin (Mb). Nobbs, Watson and Kendrew (1966) studied deoxymyoblobin by the x-ray diffraction technique at the 2.8 $\mathring{A}$ level. Within limits imposed at this level of resolution they found that the conformation of deoxymyoglobin is the same as that of oxidized myoglobin. However,

in deoxymyoglobin there is no water molecule or other group at the sixth coordination position.

In the oxygenated form the heme has a divalent iron atom which has its sixth coordination position occupied by molecular oxygen. This form is referred to as oxymyoglobin (MbO_2). When the iron atom in the heme is oxidized to the trivalent state, the heme-protein is called ferrimyoglobin or metmyoglobin (MetMb). In this form the sixth coordination position of the iron is bonded to a water molecule.



Myoglobin is a monomer with a molecular weight of approximately 17,000. Its single polypeptide chain consists of 153 amino acids linked to an iron complex of Protoporphyrin IX.

Heme is composed of four pyrrole rings linked by four methene groups. Eight of the hydrogens on the pyrrole rings are substituted by three types of side chains: methyl, vinyl and propionic acid. The iron occupies a central position in the ring. Hexa coordination of the iron atom is assumed in iron-porphyrin compounds. Four of the bonds lie in the plane of the ring and are equally bound to the pyrrole nitrogens. The other two bonds are available for attachment to other groups and lie perpendicular to the plane of the ring.

Edmundson and Hirs (1961, 1962a, 1962b, 1962c) and Edmundson (1965) chemically determined the complete sequence of amino acid residues in sperm whale myoglobin. This analysis revealed that the myoglobin molecule

contains 153 amino acid residues and possesses a molecular weight of 17,816.

Crystalline myoglobin

Kendrew et al. (1958), Bodo et al. (1959), Kendrew et al. (1960), Kendrew et al. (1961) and Kendrew (1963) used x-ray diffraction crystallography to resolve the three-dimensional structure of sperm whale myoglobin. The 2Å resolution and the partially completed 1.4 Å resolution confirmed independently the results obtained by Edmundson and Hirs. Furthermore, the results provided the first information on the complete tertiary structure of a protein. This study revealed the spatial conformation of all 1260 atoms, other than hydrogen, which make up the myoglobin molecule. Of the 153 residues in the molecule, 118 of them, or 77%, are involved in eight right-handed alpha-helical segments which make up the straight segments of the chain. There also are eight non-helical regions; seven of these are interposed between helical segments and one is at the carboxyl end of the chain. All four proline residues correspond to the corners in the chain.

The polypeptide chain is tightly wound around the heme portion giving the whole molecule a spherical appearance. There are no disulfide bridges or sulfhydryl groups stabilizing the structure. Stabilization of the molecule seems largely due to hydrophobic interaction of non-polar side chains. The structure seems compact with almost no liquid inside. The interior of the molecule is largely hydrophobic. About 30% of the polar and 45% of the non-polar side chains are directed to the inside. Some 70% of the polar side chains of the amino acid residues are directed to the out-

side. Two notable exceptions are the nitrogens of the histidine residues F-8* and E-7* which are in contact with the heme. The fifth coordination position of the iron in the heme is linked to the 3-nitrogen of the proximal F-8 histidine residue. In metmyoglobin the sixth coordination position of the iron binds water which, in turn, is hydrogen-bonded to the 3-nitrogen of the distal E-7 histidine residue. The E-7 histidine is located on the opposite side of the heme from F-8 and approximately 9 Å removed from the heme iron (Stryer et al., 1964). In the crystal, however, the E-7 histidine residue also binds to a sulfate ion. The two polar propionic acid chains of the heme stick out toward the surface of the molecule; one is linked to the CD3 arginine.

All polar groups on the surface including the peptide CO and NH groups interact either with nearby groups or else with water molecules. Of the 20-30 sulfate ions contributed by the ambient solution there are only two sulfate ions bound to the crystal; one is bound to the 1-nitrogen of the E-7 histidine (Stryer et al., 1964), the other is bound to the peptide NH groups of the E-2 and E-3 residues.

Three of the 12 histidine residues are on the inside of the molecule. Five are completely exposed to the solvent and four are partially exposed.

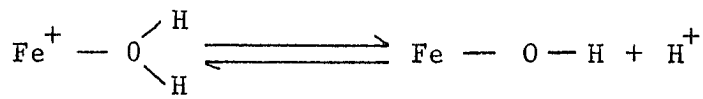
---

*Notation used by Kendrew et al. (1961) to designate the position of specific amino acids. Single letters, e.g. F or E, were used to designate helical segments, starting with A at the amino end of the molecule and progressing to H at the carboxyl end. Double letters, e.g. EF, were used to designate the non-helical segment lying between the two helices that are designated by the single letters. Arabic numerals following each letter, or combination of letters, designate the position, in sequence, of an amino acid in that particular segment of the chain, starting with 1 at the amino end.

Myoglobin in solution

The x-ray crystallographic determination of the three-dimensional structure of sperm whale myoglobin and the concomitant chemical analysis of the sequence of amino acids in the chain made it desirable to obtain information on this molecule in solution. Since all the work in this and other studies on the microheterogeneity of the myoglobin molecule were conducted on the protein in solution, it is of importance to review the facts known about its properties in solution, as well as the facts known about the myoglobin crystal.

The stability of myoglobin in solution over a wide range of pH's was well documented by Theorell and Ehrenberg (1951) and George and Hanania (1952). There is virtually no change in the spectrum of this protein from about pH 4.5 to slightly over 7.0. Below pH 4.5 there is an abrupt and drastic lowering of the Soret peak (409 nm). At pH values above 7.0 George and Hanania showed that there is a gradual decrease in the Soret peak due to the dissociation of a hydrogen ion from the water molecule that is bound to the heme.



Theorell and Ehrenberg found the pK of this dissociation to be 8.84 for horse heart metmyoglobin. Breslow and Gurd (1962) found the pK to be 8.90 for sperm whale metmyoglobin.

Beychok and Blout (1961) measured the optical rotatory dispersion of sperm whale metmyoglobin and reported 75 to 80% helical content for the protein in solution. Urnes, Imahori and Doty (1961) concluded that this molecule contains about 73% of right-handed alpha-helix. Within

experimental and computational uncertainty, these values are comparable to the 77% alpha-helix determined by Kendrew et al. (1961) through x-ray diffraction studies of the myoglobin crystal. Samejima and Yang (1964) found that sperm whale metmyoglobin and its four derivatives (ferro-, carbonmonoxy-, oxy- and cyan-myoglobin) had the same rotatory properties, indicating the absence of drastic conformational changes in the protein portion accompanying formation of the complexes.

Beychok, De Lozé and Blout (1962) found that the acid-denatured protein had a helical content of 10 to 30% depending on the conditions of denaturation, namely, pH and ionic strength. Beychok and Steinhardt (1960) had shown that the velocity of regeneration of acid-denatured ferrihemoglobin depended upon the pH and temperature at which the protein was denatured. Intrinsic viscosities of the denatured products were found to increase with decreasing pH, ionic strength and temperature. Under constant regeneration conditions they found that the greater the intrinsic viscosity of the denatured protein, the more rapid the regeneration velocity constant. Yet, helical content was shown to decrease with decreasing pH and ionic strength. It is not yet understood why the velocity of regeneration is greater for molecules possessing lower helix contents, or why regeneration rate constants, in a constant environment, depend on the denatured state. However, it cannot be concluded that residual helix content is unnecessary for regeneration; even at very low pH's Beychok and Steinhardt found a small residual helix content was retained.

Harrison and Blout (1965) suggested that the amino acid sequence alone is sufficient to determine the conformation assumed by the protein in solution. This hypothesis was based on results of studies on the

conformational changes of myoglobin and apomyoglobin in solution. They found that heme removal resulted in a 20% loss of helical structure. Complete loss of helical structure was caused by 8 M urea; this loss was completely recovered by dialysis against water or buffer. Recombination of the heme and apoprotein resulted in full recovery of native myoglobin helical content.

Similarly, Breslow et al. (1965) reported changes in optical rotation of sperm whale metmyoglobin following removal of the heme group. This indicated that the apoprotein has a different conformation than metmyoglobin. Such changes in conformation were also indicated by Breslow (1962, 1964a) and Banaszak et al. (1963b). They found that removal of heme from sperm whale myoglobin resulted in changes in side chain reactivity to hydrogen ions. Titration curves showed that at least three imidazoles are released into H^+ ion equilibrium upon removal of heme from myoglobin. Eleven imidazoles are carboxymethylated after heme removal as compared with eight imidazoles in native myoglobin. The one unreactive imidazole in globin was identified as the heme-propionate-linked imidazole in metmyoglobin (Breslow, 1964a). Large differences in tyrosine ionization between apomyoglobin and several myoglobin derivatives were also demonstrated. Similarly, Williams (1966) showed that two tryptophyl residues in metmyoglobin appear to be partially exposed to selected spectral perturbants; whereas both residues are completely exposed in acid-denatured metmyoglobin.

Changes in imidazole and tyrosine reactivity upon removal of the heme from myoglobin led Breslow (1964a) to suggest that binding of an apoprotein to its prosthetic group could lead to conformational changes in regions of the protein not necessarily proximal to the binding site.

Breslow and Koehler (1965) tried to determine whether the properties of myoglobin could be restored in the absence of the heme iron. They found that the alpha-helical content of the interaction product of Protoporphyrin IX and globin was the same as the alpha-helical content of metmyoglobin. This suggested that the binding of the Protoporphyrin IX ring, rather than the protein-iron linkages, determines the conformational differences between globin and metmyoglobin.

Breslow and Gurd (1962) showed that there is a very rapid uptake of six hydrogen ions per molecule of protein when myoglobin is titrated with hydrogen ions beginning at pH 4.5; i.e. there is only a small change in pH of the solution as these protons are added. Analysis of the titration curves indicated that the groups that become available abruptly at pH 4.5 or below are imidazole groups of histidine side chains which were previously buried due to the native conformation of the molecule.

Supporting evidence for the number of "buried" and exposed histidine groups was obtained by another approach. Nucleophilic groups catalyze the hydrolysis of p-nitrophenyl acetate to p-nitrophenol and acetic acid. Koltun et al. (1958, 1959 and 1963) showed that this method can be used to determine the number of such groups that are available on any given protein or peptide. Studies over a wide range of pH values can show which of the following groups are involved: alpha amino, epsilon amino, imidazole or phenol groups. Using this technique Breslow and Gurd (1962) showed that about six imidazoles catalyzed the hydrolysis of p-nitrophenyl acetate at pH 4.5 or above but nearly all twelve histidines were involved when the protein was denatured. However, upon returning to pH values near six after denaturation, the rate of hydrolysis was greatly increased over that

of native protein. This was taken to indicate that the polypeptide chain does not completely regain its native conformation when brought to neutrality after hydrogen ion titration.

Banaszak et al. (1963a) classified reactive and unreactive histidyl groups on the basis of alkylation of metmyoglobin with bromoacetate at pH 7.0. This reaction results in the carboxymethylation of eight histidine residues. Banaszak and Gurd (1964) showed that the four histidines which remained unreactive were C-1, F-8, FG-3 and EF-5. Carboxymethylation is a non-reversible reaction whereas hydrogen ion titrations and the p-nitrophenyl acetate reaction are reversible. Banaszak et al. (1963a) explained the two additional reactive histidine residues on this basis. Because the alkylation is not reversible, a slight change in conformation that temporarily exposes the two histidines will result in the irreversible carboxymethylation but will be reversed during the hydrogen ion titration or p-nitrophenyl acetate reaction.

Further studies by Banaszak et al. (1963b) showed that all lysyl residues in metmyoglobin are exposed and reactive. This was shown by guanidination of lysyl residues with O-methylisourea to yield homoarginyl residues without disruption of the peptide linkages.

Binding of metal ions

Breslow and Gurd (1963) showed that the binding of zinc and cupric ions to metmyoglobin resulted in the lowering of the pH of the solution. This indicated the displacement of hydrogen ions by the metals, showing that they competed for the same binding site on the protein. At a given pH zinc was found to displace fewer hydrogen ions than copper. This

indicated that zinc is a weaker competitor than copper for the binding sites on the protein. Furthermore, cupric ions were found to displace zinc ions after the latter were bound; this indicated competition between the two metals for the same sites.

Breslow and Gurd (1963) also performed titration studies on myoglobin in the presence of metal ions. The results indicated that imidazole groups took part in the chelation of cupric ions. However, on the basis of catalyzing the hydrolysis of p-nitrophenyl acetate, the same number of histidines were reported exposed as in the absence of cupric ions. This indicated that the cupric ions were either bound to histidine residues that were normally "buried" or that the normally "buried" histidine residues became exposed upon binding to the metal.

Breslow and Gurd (1963) and Breslow (1964b) showed that one or two more protons are released by the binding of each cupric ion than could be accounted for by the imidazole alone. It was shown that these protons were not released by epsilon-amino groups of lysine; no change was produced in the titration of guanidinated myoglobin in the presence of cupric ions where the epsilon-amino group was replaced by guanidinium groups. After the binding of one or two cupric ions to each myoglobin molecule there was a considerable change in the solubility of the protein-metal complex. Also, there was a drastic decrease in absorption at the Soret peak (409 nm), and a slight increase in absorption at 370 nm. These changes were similar to those observed upon acid-denaturation and suggested a change in linkage to the imidazole group of the F-8 histidine residue.

The above evidence suggests the probability of conformational changes upon binding of metal ions to the protein. Cann (1963, 1964a, 1964b)

concluded, on the basis of spectral analysis alone, that conformational changes are produced in myoglobin upon binding with zinc. He found that these large changes in the ultraviolet and visible absorption spectra are completely reversible. These differences were similar to the spectral changes that occur with acid-denaturation of the protein, the major difference being in the wavelength of maximum absorption. With zinc-reacted myoglobin this occurs at 390 nm whereas with acid-denatured protein it occurs at 370 nm. He found that zinc is bound to two ionizable groups on the protein causing concomitant conformational changes. The decrease in Soret absorption was interpreted as resulting from the rupture of the otherwise inaccessible iron-imidazole linkage and occupancy of the imidazole by zinc ion. Supporting evidence of this was found in the fact that carbonmonoxymyoglobin is very much less reactive toward zinc than metmyoglobin. Also, zinc-reacted ferro-proteins are very sensitive to oxidation.

Binding of other ions

Gillespie et al. (1966) found that incompletely deionized preparations of sperm whale myoglobin were much more resistant to denaturation by cupric ions than preparations that were completely deionized. Dialyzed preparations generally reached a pH of about 7.2. Deionized preparations varied between 7.80 and 7.95. This indicated the binding of some anion by sperm whale myoglobin. Breslow and Gurd (1962) had found that a shift in pH from 7.2 to 7.9 corresponded to a titration of nearly two units of charge.

Stryer, Kendrew and Watson (1964) had shown by x-ray crystallography

that the binding of an azide ion by sperm whale myglobin is accompanied by the release of the sulfate ion. The azide ion displaces the water molecule at the sixth coordination position of the heme iron, and there is a concomitant loss of a sulfate ion bound to metmyoglobin 9 Å from the azide site. The sulfate ion is bonded to the distal histidine residue E-7 and the arginine residue at CD3. In metmyoglobin, one of the E-7 histidine nitrogen atoms is hydrogen-bonded to the water molecule at the sixth coordination position, while the other is hydrogen-bonded to the sulfate ion. Gillespie et al. (1966) hypothesized that phosphate might be bound to the E-7 histidine side chain in the crystalline protein structure. They reasoned that phosphate and sulfate have very similar size, shape and electron density. It would, therefore, be conceivable that the protective effect of phosphate ion in solution would depend on its binding to the site attributed to sulfate in the crystalline structure. A phosphate ion in that position would greatly minimize exposure to attack by cupric ion. This tentative proposal is similar to suggestions of Cann (1964a, 1965) concerning denaturation by zinc ions.

The Microheterogeneity of Proteins

Evidence for the microheterogeneity of proteins began to accumulate in the early 1940's as techniques and instruments became increasingly sensitive. Proteins that were previously assumed homogeneous were shown to exhibit minor differences when subjected to sensitive analytical methods such as ultracentrifugation, chromatography, electrophoresis, immunological reactions and the study of amino acid sequences. Some proteins seemed "pure" by one method but heterogeneous by another. "Purity" of a protein

became a relative concept signifying no demonstrable heterogeneity by available methods.

Synge (1949) coined the term "microheterogeneity" and suggested that proteins, like peptides, might occur in "families". Colvin, Smith and Cook (1954) reviewed reports of the microheterogeneity of proteins covering the period 1946-1953. Their definition of microheterogeneity, based on Synge's concept, was

> ...a protein preparation will be said to be microheterogeneous if there is experimental evidence for one or more minor differences between individual protein molecules of the preparation, over a period which is long compared with the duration of the experiments.

They listed twenty-three different proteins that were found microheterogeneous by one method or another. Specific causative factors were given in only a few instances:

Perlmann (1949, 1952, 1953) showed by enzymatic dephosphorylation that ovalbumin is a mixture of at least three electrophoretic forms which differ by zero, one or two phosphate groups per molecule. She showed that this microheterogeneity existed in ovalbumin as obtained from the hen's egg as well as after crystallization.

Harfenist (1953), and Harfenist and Craig (1952a, 1952b, 1952c) showed that the two major components of crystalline insulin from beef pancreas, which possess equal biological activity, differ by a single amide group.

Paleus and Neilands (1950) showed that the three components obtained from chromatography on Amberlite IRC-50 of cow heart cytochrome c differed in iron content.

The review by Colvin et al. (1954) prompted re-examination of many

so-called "pure" preparations of proteins. Reports on the microheterogeneity of proteins published since 1954 are too numerous to review here. Suffice it to say that some causes for microheterogeneity have been found to range from configurational changes to differences in primary structure, such as differences in sequence of amino acids, differences in prosthetic groups or polymers of different units (Feeney, 1964, p. 352).

Among the more interesting observations on microheterogeneity is the report by Aoki and Foster (1956) that bovine serum albumin consists of three components in the region of the isoelectric point. They demonstrated that this heterogeneity is mainly due to a pH dependent transition of the normal form of the protein into a faster migrating form, presumably of a higher positive charge. The ratio of fast to slow forms was shown to increase regularly with decreasing pH. Electrophoretic results indicated that the equilibrium reaction is slow. Sogami and Foster (1963) explained the microheterogeneity of bovine serum albumin on the basis of differences in the secondary and/or tertiary structure, possibly in the detailed pairing of the half-cysteine residues in the disulfide bridges. They suggested that these differences resulted in multiple forms of the protein which are capable of undergoing a transition or conformational change in acid solution.

The microheterogeneity of myoglobin

In 1932 Theorell first succeeded in crystallizing myoglobin from horse heart. This pigment was previously referred to as muscle hemoglobin. The earliest report on the microheterogeneity of myoglobin came from Schmid (1949). He observed two or three boundaries in the electrophoresis

of crystallized finback-whale myoglobin between pH 4 and 11.

This discovery led Theorell and Åkeson (1955) to re-examine their crystallized horse myoglobin. They reported three electrophoretic components by the moving boundary technique. Lewis (1954), while working in Theorell's laboratory, had found evidence of this inhomogeneity in crystalline horse myoglobin by his acid-acetone splitting test. He determined spectrophotometrically the amount of hemin cleaved after addition of a definite amount of myoglobin of known pH to acetone. The amount of hemin cleaved was calculated from the optical density readings and these values were plotted against the pH of the solution. A slight break was noticed in the pH 4.5 region showing less than 10% cleavage for metmyoglobin. Lewis though that this was probably due to the presence of other substances seen on electrophoresis.

Bovine myoglobin Lewis and Schweigert (1955) reported three electrophoretic components of approximately 80-20-1% proportions in crystalline beef myoglobin. These three components were found on paper as well as free boundary electrophoresis. They found no difference in these components in ultracentrifugal studies. However, no importance was attached to this since they were unable to separate a mixture of hemoglobin and myoglobin in the ultracentrifuge.

Quinn, Pearson and Brunner (1964) separated bovine myoglobin into three fractions (I, I_A and II) on Carboxymethyl Cellulose (CM-Cellulose) columns at pH 6.9. They were unable to resolve these fractions further on rechromatography at pH 6.4 or 8.5. Based on 280/525 nm ratios they found the first fraction contaminated with colorless protein.

Each of the two major CM-Cellulose fractions was further resolved into two broad diffuse bands on DEAE-Cellulose columns equilibrated with 0.02M tris buffer, pH 8.3-8.4. However, on DEAE-Cellulose equilibrated with 0.02M tris buffer, pH 8.0 or 7.8, the two major CM-Cellulose fractions I and II were further resolved into 6 and 4 fractions respectively. Several of these bands disappeared with the use of 0.001M KCN. Quinn et al. (1964) concluded that this confirms the conclusion of Perkoff et al. (1962) that myoglobin components which differ only in the state of the iron can be resolved on column chromatography. Both investigators reported that all fractions obtained from chromatography were heterogeneous on electrophoresis. They obtained a total of four electrophoretic components from the CM-Cellulose fractions with the discontinuous buffer system of Poulik (1957) and the starch gel technique. However, they obtained only three electrophoretic components from "crude", $(NH_4)_2SO_4$ fractionated myoglobin.

Quinn and Pearson (1964) found no differences in the absorption spectra and autoxidation rates of the three electrophoretically and chromatographically distinct myoglobin fractions obtained from beef muscle. They did, however, find a difference in light absorbance values and susceptibility to acid cleavage for the three myoglobins. They drew the tentative conclusion that structural variations existed at the porphyrin-globin linkages.

DuFresne (1964) found that bovine myoglobin could not be chromatographed on CM-Cellulose columns using the method of Åkeson and Theorell (1960). Though Quinn et al. (1964) used this method, DuFresne found that bovine myoglobin had little affinity for CM-Cellulose at pH 6.9 and moved

with the solvent front. At pH 6.0 to 6.6, she resolved bovine myoglobin into three fractions F_1, F_2 and F_3. These fractions were preceded by a colorless, ultra-violet absorbing material not further identified. She observed that F_2 and F_3 accounted for 98% of the total myoglobin eluted but that F_2 varied greatly in size with different preparations. Column dimensions and size of the sample chromatographed were not included in her tabulation of results. Based on statistical analysis of beef heart myoglobin separated on CM Whatman paper strips, she concluded that the great variations in size of F_2 resulted from differences between two animals. DuFresne used myoglobin obtained between 40 and 100% saturation with $(NH_4)_2SO_4$. Okubo (1963) showed that though myoglobin began to precipitate at 60% saturation, these fractions contained many sarcoplasmic proteins. He showed that only the fraction obtained between 90-100% saturation was virtually free of hemoglobin. Myoglobin fractions obtained between 70-80% and 80-90% saturation contained hemoglobin.

Horse myoglobin The first observation on the heterogeneity of horse myoglobin by Theorell and Åkeson (1955) showed that the three electrophoretic components could not be separated by repeated re-crystallization of the protein. Only the two major components Mb I and II were homogeneous by electrophoresis.

In 1960 Åkeson and Theorell used an improved procedure to isolate three homogeneous myoglobin components. Ten grams of myoglobin purified by $(NH_4)_2SO_4$ fractionation was chromatographed on a 6.5x 20 cm CM-Cellulose column equilibrated with 0.02 M phosphate buffer, pH 6.9. Colorless protein emerged almost immediately from the column, followed by two broad

bands of myoglobin. The faster column component consisted mainly of the electrophoretic component Mb II with some Mb I and III, Mb I being the major electrophoretic component, Mb III the minor fastest-moving component. The slower column fraction was almost all Mb I, the slow electrophoretic component, with some Mb II still present.

The slow, most positively charged column fraction was rechromatographed on CM-Cellulose at pH 6.4. This resulted in the separation of two components: the slow column fraction was equated with the electrophoretic Mb I, the fast column fraction with electrophoretic component Mb II. When a colorless zone of 3-4 cm separated the two fractions on the column, the cellulose was pushed out of the column and the fractions were cut out and eluted. Electrophoresis at pH 6.0, 8.0 and 9.8 showed that Mb I migrated as a homogeneous protein. On rechromatography Mb II, the most negatively charged fraction, was resolved into three fractions that were equated with Mb III, II and I in order of their emergence from the column. These were precipitated with ammonium sulfate and subjected to electrophoresis. None of them were electrophoretically homogeneous. Upon repeated separation on DEAE-Cellulose and CM-Cellulose Theorell finally resolved these mixtures into two components that were electrophoretically homogeneous at pH 6.0, 8.0 and 9.8. The third component Mb III was apparently lost on rechromatography. Crystallization of the two homogeneous components Mb II_1 and II_2 showed that their crystals had the same shape as Mb I.

Analysis of the three homogeneous myoglobins showed that they had the same iron and sulfur content. No significant differences were found in their amino acid composition. Light absorption spectra were identical with only small differences at 235-280 nm, and the three myoglobins could not

be separated by ultracentrifugation. Total amide content for all three myoglobins was the same and the N-terminal amino acid was glycine. Porter and Sanger (1948) had shown that glycine was the N-terminal amino acid in unseparated horse myoglobin. Åkeson (1962) further showed that the three fractions from horse myoglobin have the same C-terminal amino acids: Leu-Phe-Glu(NH_2)-Gly(COOH).

Åkeson and Theorell (1960) further analyzed their column fractions by tryptic digest and the "fingerprinting" technique introduced by Ingram (1956, 1958). They obtained slightly more than the expected 22 peptides from each fraction. However, the fingerprints for all three were identical except for finding two additional peptides in Mb II_2, one additional peptide in Mb I and no additional peptides in Mb II_1.

Vesterberg and Svensson (1966) developed a method of isoelectric focusing by electrophoresis which they applied to isoelectric fractionation of horse myoglobin. They claimed an accuracy of $\pm$ 0.02 units in determining isoelectric points. Vesterberg (1967) determined the isoelectric points of horse myoglobin fractions purified by the method of Åkeson and Theorell (1960). He reported the following isoelectric points for these metmyoglobin fractions: Mb I, pI 7.76; Mb II_1, pI 7.32; Mb II_2, pI 7.26. He also found that Mb III, the fast-moving component from a CM-Cellulose column, consisted of three components with different isoelectric points: Mb III_1, pI 6.89; Mb III_2, pI 6.85; Mb III_3, pI 6.80.

Rat myoglobin Åkeson et al. (1960) reported a study on rat myoglobin performed in vivo. They injected the radioisotope C^{14} into rats and were able to show the existence of two species of myoglobin molecules

in vivo. The main population of myoglobin was shown to have a half-life of 80-90 days, whereas the second, short-lived myoglobin molecule had a half-life of 20 days. This seems to be the only evidence of more than one type of myoglobin molecule in the living system.

Human myoglobin It is well established that adult (α_2-β_2) and fetal hemoglobin (α_2-γ_2) have identical α-chains but the β-chain of the adult differs in primary structure from the γ-chain of fetal hemoglobin (White, Handler and Smith, 1964).

Interest in the microheterogeneity of human myoglobin stems partially from the controversial claim of the existence of a fetal form of the protein. Jonxis and Wadman (1952) claimed to have demonstrated a fetal form of myoglobin in calf heart. Singer, Angelopoulous and Ramot (1955) claimed to have demonstrated fetal myoglobin in heart and skeletal muscle of human fetuses. Perkoff and Tyler (1958), Perkoff (1964, 1965, 1966), Whorton, Hudgins and Connors (1961), Whorton et al. (1963), Benoit, Theil and Watten (1963, 1964), Theil and Williams (1967), Miyoshi et al. (1963) presented evidence for the existence of fetal myoglobin and for its occurrence in various muscle diseases. Evidence against the existence of fetal myoglobin has been presented by Rossi-Fanelli et al. (1959), Schneiderman (1962), Kossman, Fainer and Boyer (1964) and Timmer et al. (1957).

Wolfson et al. (1967) extracted myoglobin from adult and fetal muscle by two different techniques. The chromatographically separated heme proteins yielded identical electrophoretic patterns. However, the electrophoretic pattern of myoglobin from adult muscle was different from that of fetal muscle when myoglobin was extracted with 3 M phosphate buffer. They

found that the fast-moving electrophoretic component previously reported as fetal myoglobin by others resembled fetal hemoglobin by the following criteria: gel filtration, antigenicity, absorption spectra and peptide mapping. They found that fetal hemoglobin is soluble in 3 M phosphate buffer but adult hemoglobin is insoluble. Since all reports of fetal myoglobin resulted from studies of myoglobin separated from hemoglobin in 3 M phosphate buffer, Wolfson et al. concluded that the fetal myoglobin is really fetal hemoglobin. This was confirmed with peptide maps of tryptic digests of fetal muscle heme pigment and fetal hemoglobin. Theil and Williams (1967) made a similar independent observation. Based on absorption spectra in the visible light range they reported that fetal myoglobin bore a greater resemblance to hemoglobin than to adult myoglobin.

Perkoff et al. (1962) separated adult human myoglobin into four heme fractions that differed in color. These fractions were preceded by one non-heme fraction on DEAE-Cellulose at pH 7.85. They called the major fractions F_1 (brown), F_2 (dark red) and F_3 (reddish brown) in order of their emergence from the column. When F_1 and F_2 were rechromatographed under the same conditions, two components were obtained from each fraction. They suggested that the myoglobin in each fraction can be converted into the other chromatographic forms. However, rechromatography of F_1 and F_2 as oxymyoglobin and cyanmetmyoglobin resulted in the elution of one peak. The multiple electrophoretic components in F_1 and F_2 were also eliminated in the cyanmet myoglobin form. Tryptic digests of F_1 and F_2 gave identical two-dimensional peptide patterns. Perkoff et al. concluded that F_1 and F_2 were acid and alkaline metmyoglobin.

F_3, the most negatively charged fraction, yielded six components on

rechromatography. F_3 also had a greater electrophoretic mobility than F_1 and F_2. This fraction (F_3) contained significant amounts of non-heme protein which could not be completely separated on rechromatography. Analysis of tryptic digests of F_3 revealed a small difference in peptides from F_1 and F_2 but results were inconclusive due to the contaminating protein.

Whereas Perkoff et al. (1962) decreased multiple myoglobin bands on DEAE-Cellulose by using the oxy- and cyanmetmyoglobin forms, Wolfson et al. (1967) claimed to decrease the multiple bands to one component by using 2-mercaptoethanol in the agar gel on electrophoresis. The latter finding is surprising in view of the fact that human myoglobin lacks both cysteine and cystine and consequently has no disulfide bridges.

Seal myoglobin Rumen (1959) separated seal myoglobin into five fractions on CM-Cellulose columns at pH 8.5. She found that the rechromatographed column fractions were homogeneous by moving boundary electrophoresis. The isoelectric point, determined by moving boundary electrophoresis, was near pH 8.05 for myoglobin I, the major component. Myoglobin II had an isoelectric point near pH 7.6. Due to lack of protein, the isoelectric points for myoglobin III, IV and V were not determined. Rumen found glycine to be the N-terminal group of all five components. Kendrew et al. (1954) had shown that glycine was the N-terminal amino acid of unpurified seal myoglobin.

There was no difference in the minimum molecular weight of all five components. The sedimentation constant of the major component at 20°C was found to be 1.95S, a value similar to that reported by Theorell and Åkeson (1955) for horse myoglobin. All five components had the same crystals and

iron content, and showed only a slight difference in extinction coefficients.

Rumen, as others, noticed no difference in chromatograms run at 4°C and room temperature.

Rumen and Appella (1962) studied the molecular association behavior of seal apomyoglobin I, the major component from CM-Cellulose chromatography. They found a single symmetrical peak in free boundary electrophoresis at pH 8, the isoelectric point of myoglobin I. At this pH they found no change in sedimentation coefficient with different ionic strengths.

At acid pH's this protein showed a strong tendency to associate into components with a sedimentation coefficient of 4S. These 4S components could aggregate further into 8S components. Polymerization occurred between pH 3 and 5, with maximum association between pH 4 and 5. They found that the sedimentation coefficients of the 1.9S, 4S and 8S components were independent of protein concentration. However, at an ionic strength of 0.1 the relative areas under the 4 and 8S peaks were dependent on protein concentration. Increases in protein concentration increased the area under the fast 8S peak with a corresponding decrease in the area under the slow 4S peak. Rumen suggested that a slow interconversion of one component into the other might be occurring.

Optical rotation measurements indicated partial unfolding of the molecule at pH 3; consequently, the aggregation is weaker and dependent on the ionic strength of the components. Rumen and Appella thus concluded that there is some correlation between charge, conformation and aggregation of apomyoglobin I. Determination of the molecular weights of the various polymers is in progress.

Further evidence for the aggregation of myoglobin at low pH values was presented by Strausser and Bucsi (1965). They showed that human myoglobin aggregates at pH 4.5 or less. In fact, the lower the pH the more bands they obtained.

Breslow and Rumen (1967) studied the reactivity of seal myoglobins to hydrogen ions. They determined that approximately the same number of histidines are masked to hydrogen ions in fractions I and II as in whale myoglobin. However, seal metmyoglobin was found more acid-labile than sperm whale metmyoglobin. Comparison of the titration of fraction V with fractions I and II (the major components) indicated that fraction V is missing at least two histidines which are normally exposed to solvent in fractions I and II. Presumably fraction V differs in conformation from fractions I and II.

As with sperm whale myoglobin, Breslow and Rumen (1967) reported that a single tyrosine is masked in fraction I of seal myoglobin.

Whale myoglobin Myoglobin from the sperm whale has been studied more extensively than that of any other species. Its high concentration in whale muscle results in gratifying yields upon isolation. Furthermore, the complete elucidation of its three-dimensional structure at the 2 Å level by Kendrew et al. (1960) and Kendrew et al. (1961) has given investigators a unique advantage for interpreting results on the molecular level. This has made sperm whale myoglobin the protein of choice for a wide variety of studies.

Edmundson and Hirs (1962a) resolved crystalline sperm whale myoglobin into at least five components on the carboxylic resin IRC-50. They

state that Bodo and Kendrew, in a personal communication, informed them of resolving this crystalline protein into at least four fractions on the sodium-form of IRC-50. The four components representing 92% of the protein were shown to have identical amino acid compositions. The fifth component, namely the most negatively charged first fraction eluted from a pH 5.82 column, was not obtained free of non-heme protein; however, it appeared to be very similar to, if not identical with, the composition of the other heme-containing components.

Edmundson and Hirs (1962a) showed that sperm whale myoglobin is very sensitive to small shifts in pH on IRC-50 chromatography. They showed that at pH 5.92 the components moved relatively fast, with poor separation, whereas at pH 5.82 they moved much more slowly with partial resolution. Rechromatography on IRC-50 at pH 5.82 showed these fractions to be homogeneous if allowances are made for tailing.

Amino acid analysis of the non-heme component that preceded myoglobin showed that this colorless protein differed markedly in composition from the heme-containing fractions (Edmundson and Hirs, 1962a). Apparently the colorless protein is not the apoprotein from myoglobin. Atassi (1964) also found that the non-heme component that precedes sperm whale myoglobin on CM-Cellulose columns has an amino acid composition completely different from that of myoglobin. Furthermore, he reported that the non-heme protein did not react with antiserum to myoglobin.

Atassi (1964) resolved sperm whale myoglobin into eleven heme fractions on CM-Cellulose. He worked with the cyanmetmyoglobin derivative in order to eliminate differences due to acid-alkaline forms suggested by Perkoff et al. (1962). Atassi stated that he had previously found

cyanmetmyoglobin homogeneous by electrophoresis on cellulose acetate in pH 8.6 barbiturate buffer. However, he resolved cyanmetmyoglobin into four distinct components on starch-gel electrophoresis in the discontinuous buffer system of Poulik (1957).

Spectral analysis of CM-Cellulose fractions revealed that heme-components II - IV were in the met form. Fractions V - XII had typical cyanmetmyoglobin spectra.

Fractions II - XII had identical amino acid compositions which agreed well with those obtained by Edmundson and Hirs (1962a) for their major myoglobin component.

However, Atassi (1964) found that all the myoglobin fractions interconverted. Each fraction, upon standing for a few hours, was transformed into all the others. He observed this transformation by both chromatography and electrophoresis. An equilibrium involving different polymeric forms was ruled out; molecular weights of monomers were obtained for some of these components by means of sedimentation equilibrium studies.

By immunoelectrophoresis Atassi showed that the multiple lines of identity obtained by the agar double diffusion technique were probably due to the conversion of one component to all the others. The fusion of all lines obtained at their ends indicated complete antigenic similarity.

Atassi and Saplin (1966) reported similar results for finback whale myoglobin. They separated this protein into eight chromatographic heme components and one non-heme component. Interconversion of these components was found by both chromatography and electrophoresis. The chromatographic components were homogeneous by ultracentrifugation.

Hardman et al. (1966) confirmed the microheterogeneity of sperm

whale myoglobin as reported by Edmundson and Hirs (1962a). Instead of isolating myoglobin by the procedure described by Kendrew and Parrish (1956), Hardman et al. used a zinc-ethanol preparation and CM-Cellulose columns. They separated myoglobin into at least four fractions; these were preceded by a non-heme component. They were not able to resolve myoglobin into homogeneous fractions on DEAE-Cellulose. Hardman et al. reported four and sometimes five electrophoretic components from native myoglobin by the disc gel and vertical gel techniques. They found the major electrophoretic components homogeneous on re-electrophoresis and implied electrophoretic homogeneity for the two major chromatographic fraction from CM-Cellulose. However, CM-Cellulose fractions were shown to react with anti-myoglobin serum on Ouchterlony plates forming lines of identity with each other. They also reported that native myoglobin had the same number of electrophoretic components as cyanmetmyoglobin, guanidinated metmyoglobin and the apoprotein. Each band of the latter, however, migrated at a slower rate than the corresponding band in native myoglobin because of the removal of the two negative charges borne by propionic acid side chains of the heme and the partial negative charge due to the heme-bound hydroxyl ion. Hardman et al. concluded that the differences in myoglobin components probably reside in amino acid composition, sequence or both. They concurred with the suggestion of Edmundson and Hirs (1961) and Edmundson (1965) that the differences may lie in variations of total amide content for the glutamic and aspartic residues. Hardman et al. (1966) found no supporting evidence for the acid-alkaline myoglobin mixture postulated by Perkoff et al. (1962), and none of their reported results gave any indication of the interconversion of components.

Stockwell (1961) separated sperm whale myoglobin into two fractions on CM-Cellulose at pH 7.2. She called the major component fraction II. Tryptic digests of these two fractions were studied by the "fingerprinting" technique. The fingerprints were found to be identical except for the presence of one additional negatively-charged tyrosine-containing peptide in fraction I (the minor component). Amino acid analysis of the additional peptide revealed that it contained no lysine or arginine and must, therefore, represent the carboxyl-terminal peptide of the protein. This additional peptide was shown to contain glutamic acid, glycine, leucine and tyrosine. According to Edmundson and Hirs (1962a) the carboxyl-terminal peptide of sperm whale myoglobin consists of glu.leu.gly.tyr.glu-NH_2. Stockwell suggested that the substitution of glutamic acid for a glutamine in the additional peptide would account for its greater negative charge. However, evidence for such a substitution was not presented.

In conclusion, there seems to be no immediate explanation for the microheterogeneity of myoglobin. More than one fraction is obtained from myoglobin of various species on chromatography and electrophoresis. A non-heme protein has been shown to accompany myoglobin isolated by different procedures. The number of fractions obtained on chromatography and electrophoresis seems to vary with the system used. No significant differences have been found in primary structure of the different fractions. A difference in amide content has been suggested by Edmundson and Hirs (1961) and Edmundson (1965) as a possible explanation but no concrete evidence for this has been presented. Åkeson and Theorell (1960) obtained up to two additional peptides in fingerprinting horse myoglobin fractions; Stockwell (1961) found one additional peptide in fingerprinting sperm whale

myoglobin fractions. A difference in amino acid sequence would have resulted in the same number of peptides with one or more differing in mobility. The additional peptides suggest a difference in charge which might be due to a difference in amide content or sequence in some of the molecules. However, no differences in total amide content have been proven. Gillespie et al. (1966) suggested the attachment of a phosphate ion to the myoglobin molecule.

Rumen and Appella (1962) and Strausser and Bucsi (1965) showed that myoglobin tends to aggregate at pH values below 4.5. However, none of the experiments on microheterogeneity were carried out at such low pH values. Edmundson and Hirs (1962a) showed a pH dependent shift in elution curves of myoglobin. Throughout the literature, different elution curves have been reported, under various experimental conditions, for any one species. From two to eleven CM-Cellulose fractions have been reported for one species. However, no attempt has been made to correlate pH with some of the varied results obtained on chromatography and electrophoresis.

The following sections represent a study of the experimental conditions that might lead to such varying results. An attempt is made to determine the effect of pH on chromatography and electrophoresis of bovine myoglobin, and to correlate the findings with proposed explanations for the microheterogeneity of that protein.

MATERIALS AND METHODS

Materials

Laboratory reagents

Reagent-grade chemicals were used in all experiments. Water used in all procedures was deionized by passing distilled water through a mixed-bed ion exchange resin then through an all-glass still. Its electrical conductivity indicated an ion contamination, expressed as sodium chloride, of less than 0.05 PPM.

CM-Cellulose

All CM-Cellulose used in the course of this study was purchased from the Sigma Chemical Company, St. Louis, Missouri. This cellulose was medium mesh with a capacity of 0.63 milliequivalents per gram.

Methods

CM-Cellulose columns

Treatment of CM-Cellulose The cation exchanger was used in the potassium form. It was converted from the sodium form by equilibration with potassium phosphate buffer. Prior to use, all CM-Cellulose was treated in the following manner.

The exchanger was suspended in an equal volume of 0.5 M NaCl-0.5 M NaOH and left in the cold overnight. It was then washed with 3-4 liters of warm (40-45°C) deionized water on a Buchner funnel using moderate suction. The cellulose was then washed in room temperature deionized water until neutral. The preparation was then resuspended in an equal volume

of 0.5 M NaCl-0.5 M NaOH. After standing several hours with occasional stirring the CM-Cellulose was filtered and resuspended in 3-4 volumes deionized water for one hour. Decantation and washing of the cellulose with water was continued until the supernate was clear of fines upon standing for twenty minutes.

Equilibration of CM-Cellulose The filtered CM-Cellulose was suspended in 3-4 volumes of 0.1 M potassium phosphate buffer of the desired equilibrating pH and placed on a magnetic stirrer for 1-2 hours. After filtering on a Buchner funnel this process was repeated twice, or until the cellulose suspension attained the desired pH. To adjust the ionic strength the process was then repeated using three changes of 0.01 M potassium phosphate buffer of the desired equilibrating pH. The CM-Cellulose was left in the final equilibrating buffer until used.

Operation of the columns Columns of the following dimensions were used: 1.5 x 23-25 cm, 2.5 x 5-8 cm, 2.5 x 17-18 cm, 2.5 x 60 cm.

Gravity packed columns were developed at room temperature with starting pH's of 5.5, 5.9, 6.0 and 6.8. The protein was eluted from all columns by means of a pH gradient, except on pH 6.8 columns where the protein was eluted with the starting buffer. Pressure packing of columns did not alter the elution pattern.

The pH gradients were established in two different ways, both giving the same end-results:

a) 300 ml of 0.01 M potassium phosphate buffer at the equilibrating pH was placed in an Erlenmeyer flask on a magnetic stirrer. This was connected in a closed system by means of tygon tubing to another Erlenmeyer flask of

the same size and shape, containing an equal volume of 0.01 M potassium phosphate buffer, pH 7.4. The flask containing buffer of the starting pH was connected to a teflon-coated pump which, in turn, was connected to the column by means of teflon tubing.

b) Two liters of 0.01 M potassium phosphate buffer, pH 6.0, was placed in a beaker on a magnetic stirrer. This was connected to an identical beaker with a second buffer consisting of 2 liters of 0.01 M potassium phosphate, pH 8.0. The beaker containing the lower pH buffer was connected to a teflon-coated pump which, in turn, was connected to the column by means of teflon tubing. This pH gradient was used primarily for the 2.5 x 60 cm column.

A Whatman No. 1 filter paper was cut to size and placed on top of the column bed. Metmyoglobin samples between 23 and 704 mg in 5-20 ml of equilibrating buffer were placed on top of the filter paper and allowed to adsorb to the top of the column bed. A buffer head of 15-200 ml was pipetted on to the columns before starting the elution.

Myoglobin was eluted at the rate of 1 ml per minute and collected in 7-8 ml fractions. The absorbance of the fractions was measured at 280 nm and 409 nm in a Zeiss spectrophotometer. The percentage of myoglobin in fractions eluted from CM-Cellulose columns was calculated from the area under the 280 nm peak of the elution curve. The area under each peak was estimated as the area of a triangle. Spectra were recorded in the visible and ultraviolet range on a Beckman DK-2A recording spectrophotometer.

In order to compare electrophoretic patterns of eluted CM-Cellulose fractions to patterns obtained after separation of the myoglobin on the column without complete elution, a pH 6.0, 2.5 x 22 cm column was prepared

as previously described and packed under 4 lbs of pressure. The 500 mg of myoglobin was separated into four visible fractions by passing potassium phosphate buffer, pH 6.0, through the column at the rate of 1 ml per minute. When the fastest-moving fraction reached the lower part of the column, the whole column was pushed out in one piece by using 2-4 lbs pressure. The cellulose was then cut at the points of visible separation of the fractions. The myoglobin in each portion was eluted with potassium phosphate buffer, pH 6.8, 0.1 M NaCl. These samples were then dialyzed extensively against deionized water and concentrated by lyophilization for electrophoresis.

Treatment of dialysis tubing

Prior to use, dialysis tubing was boiled in 1 M sodium bicarbonate for approximately 2 hours. The tubing was then rinsed and boiled in deionized water until used.

No difference in results was observed when dialysis tubing was not treated in the above manner. Consequently, this procedure was discontinued in later experiments.

Isolation and purification of myoglobin

Myoglobin was isolated by a modification of the method of Snyder and Ayres (1961).

Ten pounds of freshly cut bovine round steak was trimmed of all visible fat and ground. The ground meat was blended for 15-30 seconds in a Waring blendor with an equal volume of deionized water. The homogenate was then centrifuged at 10,000 rpm (12,000 x G) in a refrigerated centrifuge for 15-20 minutes. The supernatant fluid was saved and the

extraction procedure repeated until the meat had lost all its red color. The pooled supernate was placed in dialysis tubing and concentrated to one-half or one-third the original volume by pervaporation.

After concentrating, the extract was heated to 55°C on a magnetic stirrer and cooled to room temperature. The precipitated heat-labile proteins were discarded after centrifugation. The pH of the supernatant fluid was adjusted to 6.5 with 5 N NaOH. The solution was then brought to 75% saturation with ammonium sulfate. After standing overnight, the extract was centrifuged and the precipitated protein was discarded. These steps were repeated bringing the solution to 85%, 90%, 95% and 100% saturation with ammonium sulfate. The extract was left at room temperature after reaching the 95% saturation point. Only the myoglobin that precipitated between 90 and 100% saturation with ammonium sulfate was used in these experiments. These fractions were free of contamination with hemoglobin. The amount of ammonium sulfate added at each step was calculated from the nomogram in Dixon and Webb (1964, p. 40).

Myoglobin isolated in this manner will be referred to throughout the text as purified myoglobin, or purified metmyoglobin.

Calculation of the relative purity of myoglobin

Ratios of the absorbancy at 409/280 nm were used as a rough estimate of the relative purity of metmyoglobin in the original sample and in the column fractions.

Calculation of the concentration of myoglobin

Absorbancy of cyanmetmyoglobin at 540 nm was used to determine the concentration of the heme protein, using an extinction coefficient of

11.3 $l\text{-}cm^{-1}\text{-}mmoles^{-1}$.

Concentration of dilute protein solutions

Myoglobin solutions were lyophilized after extensive dialysis against deionized water. No differences were found on either chromatography or electrophoresis between myoglobin that was lyophilized and myoglobin obtained by ammonium sulfate fractionation without lyophilization.

Some samples were concentrated by means of a Diaflo syringe with UM-2 membranes, made by the Amicon Corporation, 280 Binney Street, Cambridge, Massachusetts. These samples had a pH of 5.6 after being concentrated. When used in electrophoresis, they were allowed to equilibrate to the desired pH, after placement on the gel, by circulating the buffer in the cell for 10-20 minutes before turning on the current.

CM-Sephadex columns

CM-Sephadex C-50 was used according to the directions of the manufacturer.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out by the vertical gel technique described by Raymond and Wang (1960), and Raymond and Nakamichi (1962).

Polymerization of the gel, electrophoresis of the samples and destaining of the gels were all done in apparatus of E-C Apparatus Company. The acrylamide monomer and the crosslinking agent, methylene-bis-acrylamide, were purchased pre-mixed in the ratio of 95 to 5 as Cyanogum-41 from E-C Apparatus Company. The mixture was dissolved in appropriate buffers in

amounts of 5 g, 7 g, 8 g or 10 g per 100 ml buffer. N,N,N',N'-Tetramethylethylenediamine (Eastman Organic Chemicals), one of two catalysts, was added at the rate of 0.2 ml per 100 ml gel solution. The mixture was then filtered in order to remove any insoluble materials. To initiate polymerization 0.2 g per 100 ml ammonium persulfate was added. The solution gelled in the chamber cooled with tap water in about 15-20 minutes. Analytical gels were prepared with 150 ml of the appropriate buffer; preparative gels were prepared with 220 ml buffer.

Buffers used were barbital, 0.0075 M, pH 8.6; tris (0.076 M)- citric acid (0.005 M) in the gel and sample with a pH of 8.6, and boric acid (0.3 M) - NaOH (0.05 M), pH 7.9, for the buffer circulating in the cell (discontinuous system of buffers, Poulik, 1957) with and without 10^{-3}M EDTA; Poulik's discontinuous system of buffers with 0.01% w/v KCN added; Poulik's discontinuous system of buffers with 0.2 M 2-mercaptoethanol added (sp. gr. 1.1143 g/ml; Mol. Wt. 78.14 g/l; therefore, used 14.0 ml 2-mercaptoethanol per liter); 0.005 M potassium phosphate buffer, pH 6.0, and pH 6.7; 0.005 M citrate buffer, pH 5.2; and 0.0065 M potassium phosphate buffer pH 5.6; 0.1 M TEB buffer, pH 9.2 (40 g tris - 4 g EDTA - 1.52 g boric acid in 4 liters distilled water).

Lyophilized myoglobin was dissolved in the appropriate buffer and 20% sucrose added after filtration, to increase the density of the sample. In order to keep the sample in a thin band, the preferred concentration of myoglobin for analytical gels was 0.3 - 0.4 mg in a total sample volume of 0.01-0.02 ml. Samples were layered into the sample slot by means of a microsyringe. Myoglobin concentrations of 25-30 mg were used on preparative gels.

Voltages ranged from 150 to 300 volts depending on the buffer used. So that the gel would not over-heat or warp, starting amperages of 120-130 ma were not exceeded; the voltage was chosen accordingly. Length of time during which the current was applied varied from one to six hours. At the end of electrophoresis, at a constant voltage, the amperage dropped to 50-70 ma.

Staining of gels After electrophoresis, the gel slab was placed in a pyrex dish for staining. A modification of the method of Haut et al. (1962) was used for the benzidine stain which depends on the peroxidase activity of the heme group. The staining solution was freshly prepared each time before use. The following materials were combined and poured over the gel which was allowed to stain for 10-15 minutes: 50 ml methanol saturated with benzidine dihydrochloride, 10 mg sodium nitroferricyanide freshly dissolved in 50 ml distilled water, 10 ml glacial acetic acid and 3 ml of 3% hydrogen peroxide.

When duplicate samples were run, the gel slab was cut in half. One half was stained with the benzidine stain, the other half was placed in amido black dye solution for 20-30 minutes. The dye solution was made by dissolving 10 g Amido Black 10B (Hartman-Leddon Co.) in a solution containing 500 ml methanol, 500 ml distilled water and 100 ml glacial acetic acid, letting it stand overnight and filtering before using. After removing from the staining solution the gel was rinsed well with tap water then destained by electrophoresis in a charcoal-filtered bath (E-C Apparatus Co.). The destaining solvent consisted of 2 liters methanol, 2 liters distilled water and 450 ml glacial acetic acid. After destaining, the

gels were wrapped in Saran Wrap (Dow) to prevent the loss of moisture.

Photographs of gels were taken immediately after staining and destaining with a Polaroid Land Camera, Model 180. The gels were placed on a film viewer (Laboratory Supplies Co., Inc.) which was covered with a heavy black paper in which an opening had been cut out the size of the gel.

Elution of samples from preparative gels Myoglobin bands were eluted from preparative gels by two methods:

a) Gel strips for each band were cut out and placed in a mortar. Five mls of 0.01 M potassium phosphate buffer, pH 6.5 was added to each strip. The gel was then mascerated manually with a pestle and let stand overnight in the refrigerator. The preparation was then filtered and the procedure repeated. Total recovery of myoglobin by this method was poor but the extract was free of polyacrylamide gel.

b) Attempts to elute with a tissue homogenizer were unsuccessful. The preparation became pasty and impossible to recover. A similar approach was used where the gel strips were repeatedly homogenized with buffer in a small blendor then filtered. Recovery of myoglobin was good but a considerable amount of polyacrylamide gel was found in the dialyzed, lyophilized extract.

Two-dimensional electrophoresis Two-dimensional gel electrophoresis was performed in a manner similar to the technique described by Raymond and Aurell (1962). The equipment was the same as above and the discontinuous buffer system of Poulik (1957) was used. The amount of myoglobin subjected to electrophoresis on a 5% polyacrylamide gel in the first dimension was varied between 0.4 and 0.8 mg. After separation in the first

dimension two whole samples were cut out in vertical strips 5-6 mm wide. One was stained, the other placed horizontally on a fresh 8% gel for electrophoresis in the second dimension, using the same system of buffers. In order to minimize diffusion, the gel strip was kept on ice until ready to be used. A preparative 8% gel (220 ml buffer) was used for electrophoresis in the second dimension in order to accomodate the thickness of the gel strip with ease. A small slot-former, 1 cm in width, was used for electrophoresis in the first dimension, and a 10 cm wide slot-former was used for electrophoresis in the second dimension in order to accomodate the full length of the gel strip.

Nomenclature: designation of fractions

In the following presentation of results, the major CM-Cellulose fraction, i.e. the most positively charged component, will be referred to as Mb I according to the nomenclature used by Åkeson and Theorell (1960) and Rumen (1959, 1960). The minor fractions will be referred to consecutively as Mb II, Mb III etc., except for the first fraction to separate on a column. This fraction, the least positively charged, will be designated the Fast-Moving Fraction (FMF) because it seems to differ from the others in its behavior.

The major component on electrophoresis, which can be equated with CM-Cellulose Mb I, will be designated e-1. The minor components will be designated e-2, e-3, e-4 in consecutive order from e-1.

RESULTS

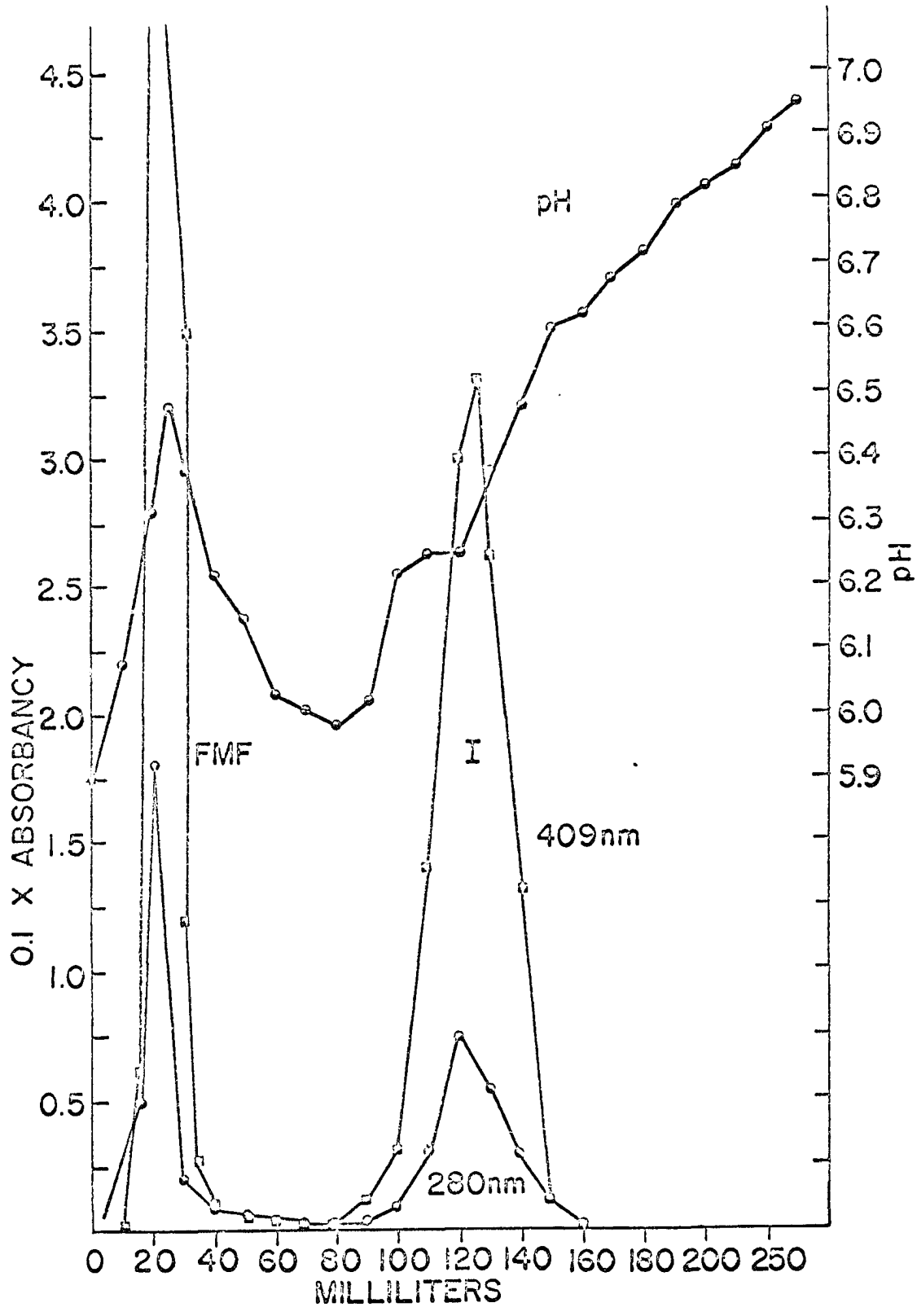
The microheterogeneity of myoglobin from various species has been demonstrated frequently by CM-Cellulose chromatography and electrophoresis. However, the number and proportion of fractions reported in the literature are not always consistent for any one species. Edmundson and Hirs (1962a) have shown a shift in elution pattern upon a slight change in pH. No explanation was offered.

The data to be presented here will show that elution patterns from CM-Cellulose columns, and the size of fractions obtained, can be altered by raising or lowering the starting pH, as well as by varying the size of a column and the amount of myoglobin used. A similar pH dependent change will be shown for electrophoretic patterns.

The Non-Heme Component

As previously cited, a non-heme fraction has been shown to precede myoglobin fractions from sperm whale, finback whale, horse and beef on CM-Cellulose columns. This non-heme contaminant is present regardless of the method of isolation and purification of myoglobin. This observation led to the speculation that the non-heme component might be a contaminating protein which, by some protein-protein interaction, is responsible for the heterogeneity of myoglobin on chromatography. The idea was further nurtured by the fact that this non-heme protein, though not demonstrable on electrophoresis of crystalline myoglobin, is demonstrable as an electrophoretic component of the Fast-Moving Fraction (FMF) from a short (2.5 x 5 cm) pH 6.0 CM-Cellulose column (See Fig. 1). The non-heme protein was more positively charged than the myoglobin components and, on

Fig. 1. CM-Cellulose chromatogram of bovine metmyoglobin. Approximately 200 mg of purified metmyoglobin in 0.01 M potassium phosphate buffer, pH 6.0, was applied to the column, 2.5 x 5 cm bed dimensions. Estimated amount of myoglobin eluted in each fraction: FMF approximately 17%; Mb I, 33%


4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.1 X ABSORBANCY
7.0
6.9
6.8
6.7
6.6
6.5
6.4
6.3
6.2
6.1
6.0
5.9
pH
pH
FMF
I
409nm
280nm
0 20 40 60 80 100 120 140 160 180 200 250
MILLILITERS

electrophoresis at pH 8.6, stayed close to the origin of the gel (i.e., the negative pole).

The non-heme protein was not electrophoretically demonstrable in crystalline myoglobin by differential staining. However, it was rationalized that, during chromatography, this protein pulled some myoglobin down the column with it and, consequently, formed a relatively large proportion of the first eluted fraction. Thus, on electrophoresis of the FMF, the non-heme component was seen as a separate band.

Electrophoresis in barbital buffer at pH 8.6 showed that neither Mb I nor the FMF was homogeneous. Mb I consisted mostly of e-1 with some e-2 and e-3, whereas the FMF consisted mostly of e-2 and e-3 with some e-1. Again, this was taken to indicate that a possible interaction of the non-heme protein with myoglobin altered the charge on myoglobin and caused a change in the proportion of the components; i.e. the non-heme protein was thought to exert an effect on some of the myoglobin.

Myoglobin was then isolated in the same manner described under "Methods" except that the pH was raised to 6.9 during the ammonium sulfate fractionation instead of being maintained at 6.3. Chromatography of this preparation yielded the same two fractions, Mb I and the FMF. However, electrophoresis of these fractions revealed that a different non-heme protein was isolated in this instance. This non-heme protein was more negatively charged and, therefore, at pH 8.6, migrated toward the positive pole more rapidly than the myoglobin components.

These experiments indicated that different contaminants were isolated with myoglobin depending on the procedure used in obtaining this protein. Furthermore, electrophoresis of the non-heme fraction showed it to be

heterogeneous.

In order to study further the effect of the non-heme component on myoglobin, an attempt was made to separate the two completely by extending the columns.

Fig. 2 shows the elution curve from a pH 5.9 CM-Cellulose column where a separation of the non-heme component was obtained. Extending the column not only separated the non-heme component but also resulted in the separation of three myoglobin fractions.

Fig. 3 shows that none of these myoglobin fractions is electrophoretically homogeneous. The FMF (Fig. 3C) still shows three electrophoretic components, but the non-heme component was not seen on the control gel.

The ratio of the absorbancy at 409/280 nm was used as a criterion of the purity of this FMF. It had a ratio of 3.2, indicating an incomplete separation of the non-heme component. Conceivably a sufficient amount of the non-heme component remained attached to the myoglobin and pulled some of it down the column, but not enough of it was left to be detected as an electrophoretic component in the FMF. This indicated a need for further purification.

Another pH 6.0 column was prepared using a highly purified myoglobin preparation with an absorbancy ratio (409/280 nm) of 4.9 before chromatography. This myoglobin was obtained from an $(NH_4)_2SO_4$ fractionation where only the fraction at 95-100% saturation was used. Under the same conditions, this preparation gave the same elution curves seen in Figs. 1 and 2. The column length was therefore, increased to 2.5 x 60 cm in order to effect a better separation.

Fig. 2. CM-Cellulose chromatogram of bovine metmyoglobin. Approximately 280 mg of purified metmyoglobin in 0.01 M potassium phosphate buffer, pH 5.9, was applied to the column, 2.5 x 17.5 cm, bed dimensions. Estimated amount of myoglobin eluted in each fraction: FMF approximately 4%; Mb II, 10%; Mb I, 86%

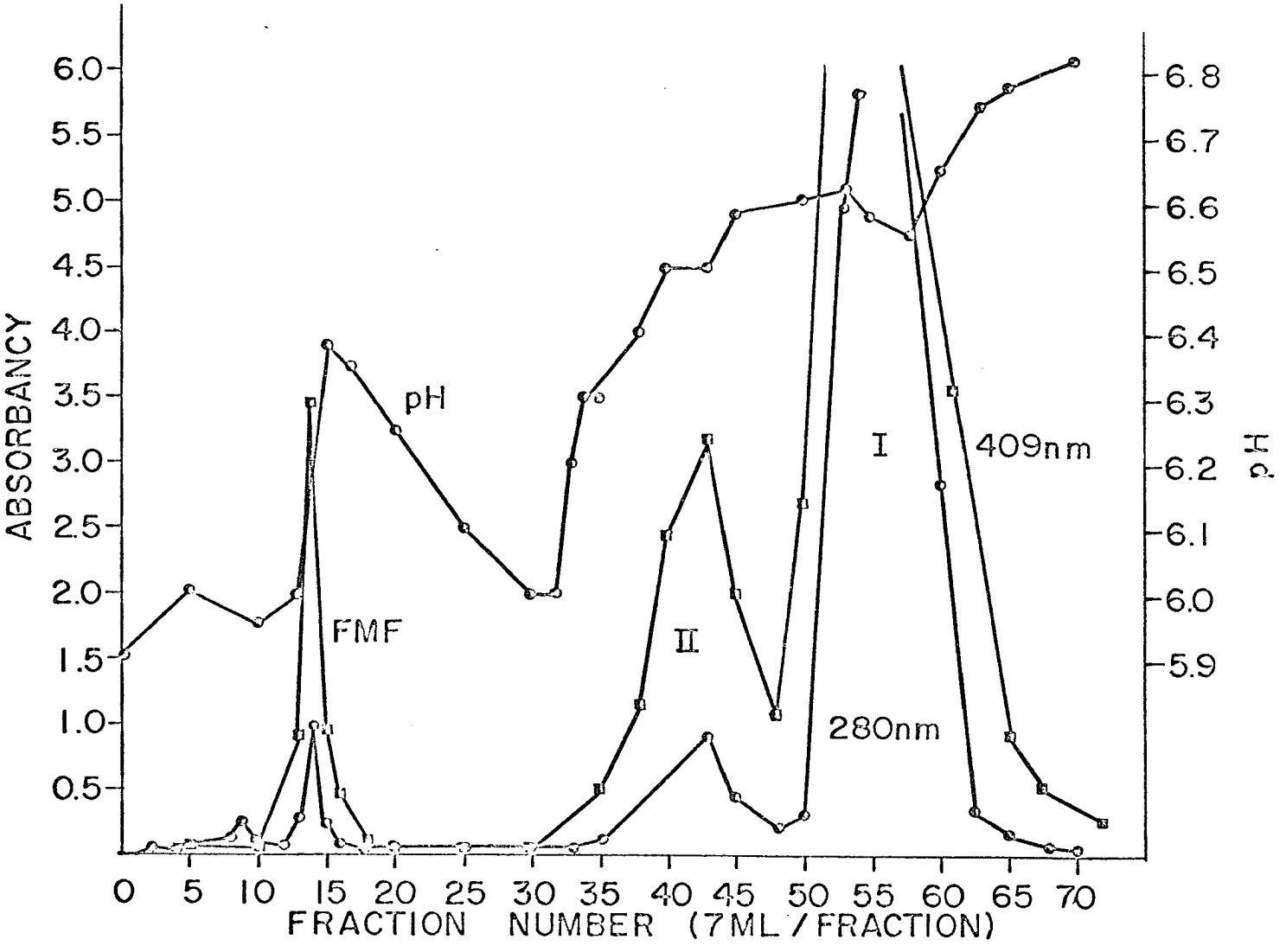

ABSORBANCY
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
pH
6.8
6.7
6.6
6.5
6.4
6.3
6.2
6.1
6.0
5.9
FMF
II
I
409nm
280nm
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
FRACTION NUMBER (7ML / FRACTION)

Fig. 3. Polyacrylamide gel electrophoresis of purified metmyoglobin, and fractions from a pH 5.9 CM-Cellulose column (2.5 x 17.5 cm), shown in Fig. 2. Electrophoresis of these samples was done in an 8% gel by the vertical gel technique, using Poulik's discontinuous system of buffers, pH 8.6. Origin of the gel is at the top; samples are migrating downward towards the positive pole. Electrophoretic bands, from top to bottom are e-1, e-2, e-3 and e-4. The samples are, from left to right:
A = Mb I
B = Mb II
C = FMF
D = Purified metmyoglobin


e-1
e-2
e-3
e-4
A B C D

Fig. 4 shows that the non-heme component emerged far ahead of the myoglobin on this column and was present in a very low concentration. Yet, the myoglobin separated into four or five fractions. It now became evident that the non-heme component could not be inducing the myoglobin fractions obtained from CM-Cellulose chromatography. However, it was also observed that the fractions from the column had a lower 409/280 nm ratio than the original sample.

It also became apparent that myoglobin from a single species could be separated into 2-5 chromatographic fractions depending on the conditions used. The reason for this was not immediately obvious. The experimental data indicated that the myoglobin fractions were not due to the non-heme component. Consequently, further experiments were designed to determine which factors were responsible for these varied results in the chromatography of myoglobin.

CM-Cellulose Chromatography

Relationship of column capacity and pH to elution patterns

Figs. 1, 2 and 4 all show elution curves of myoglobin chromatographed at a starting pH of 6.0. These columns differed only in size and the amount of myoglobin chromatographed. However, each column had a different elution pattern, differing primarily in the number and size of fractions eluted. These columns indicated the importance of column capacity.

A comparison of columns in Table 1 shows the relationship of column size, and amount of myoglobin chromatographed, to the size of the FMF. Columns A and B have approximately the same amount of myoglobin per cubic centimeter column bed. In both instances the FMF consisted of 4% of the

Fig. 4. CM-Cellulose chromatogram of purified metmyoglobin (the fraction obtained from an ammonium sulfate fractionation at 95-100% saturation). Approximately 300 mg metmyoglobin in 0.01 M potassium phosphate buffer, pH 6.0, was placed on the column, 2.5 x 60 cm bed dimensions. Estimated amount of myoglobin eluted in each fraction: Mb I, approximately 92.7%; MB II, 5%; Mb III, 1.5%; Mb IV, 0.8%

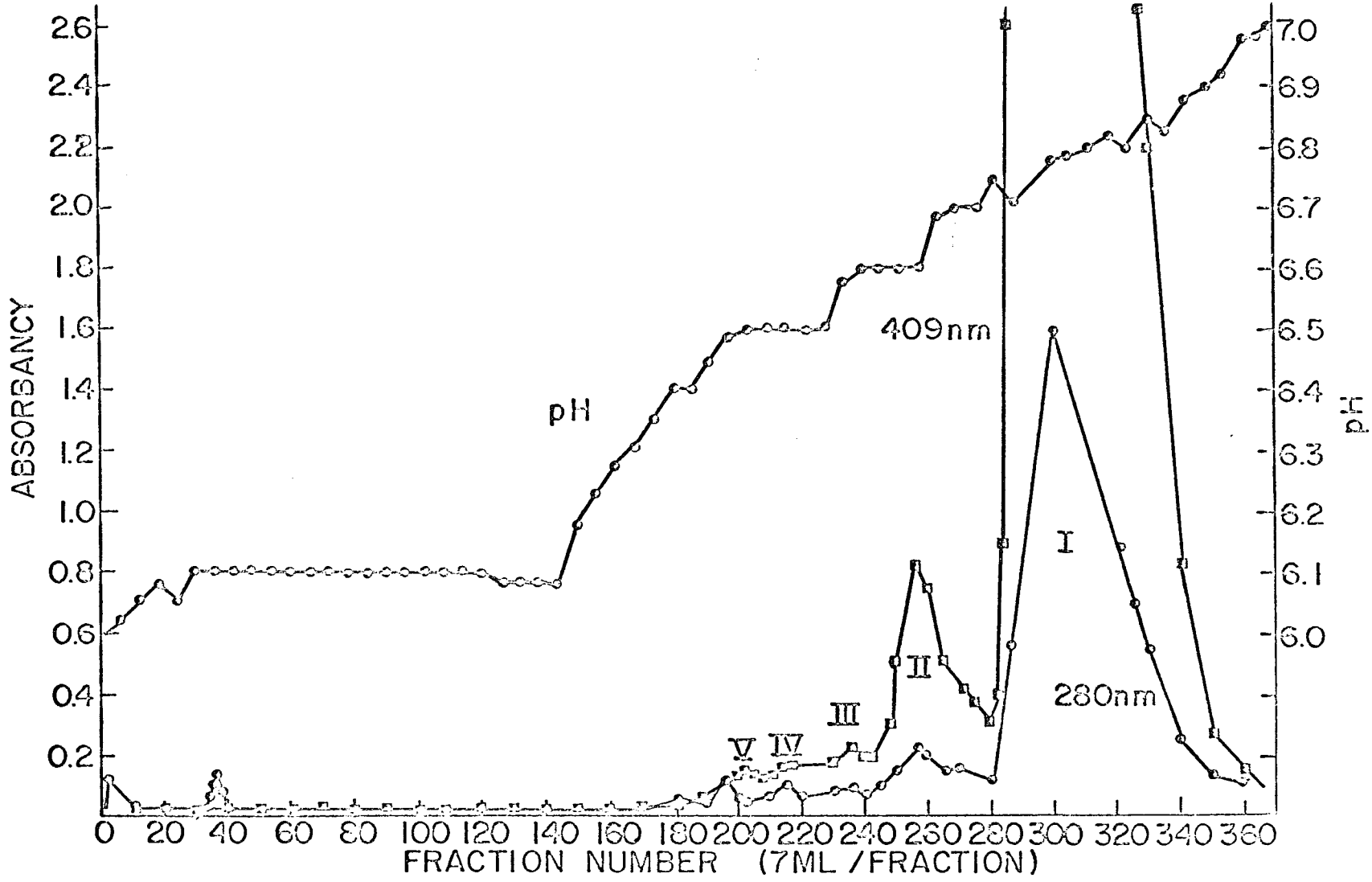

ABSORBANCY
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
pH
409nm
280nm
I
II
III
IV
V
7.0
6.9
6.8
6.7
6.6
6.5
6.4
6.3
6.2
6.1
6.0
pH
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360
FRACTION NUMBER (7ML /FRACTION)

Table 1. Relationship of equilibrating pH and column capacity to fractions eluted from CMC[a] columns

Equilibrating pH and column code		column dimensions in cm	column capacity in cc $pi(r^2h)$	mg MB chroma-graphed	mg Mb per cc CMC	Total number fractions eluted	Per cent FMF (estimate)
5.9	A	1.5 x 23	40.7	125	3.1	3	4%
5.9	B	2.5 x 17.5	85.9	280	3.3	3	4%
6.0	C	2.5 x 60	295	300	1.0	4 or 5	None
6.0	D	1.5 x 24.5	43.4	23	0.53	3 or more	None
6.8	E	1.5 x 25	44.3	22	0.49	2	7%
6.0	F	2.5 x 5	24.6	250	10.2	2	24%
6.0	G	2.5 x 5.5	27.0	206	7.7	2	17%
5.5	H	2.5 x 18	88.4	437	4.9	4	0.5%
5.5	I	2.5 x 17	83.5	704	8.4	4	0.3%
6.0	J	2.5 x 8	39.3	252	6.4	2	8%
6.0	K	2.5 x 8	39.3	174	4.4	2	2%

[a]CMC refers to Carboxymethyl Cellulose.

total myoglobin eluted. Columns A and D are similar in size; however, a much smaller amount of myoglobin was chromatographed on D than on A (mg Mb/cc column bed). Consequently, the FMF on column D equilibrated with the column instead of being eluted, whereas column A had a FMF consisting of 4% of the total myoglobin eluted. Columns F and G, and J and K, in each instance show that the column on which more myoglobin was chromatographed had the larger FMF.

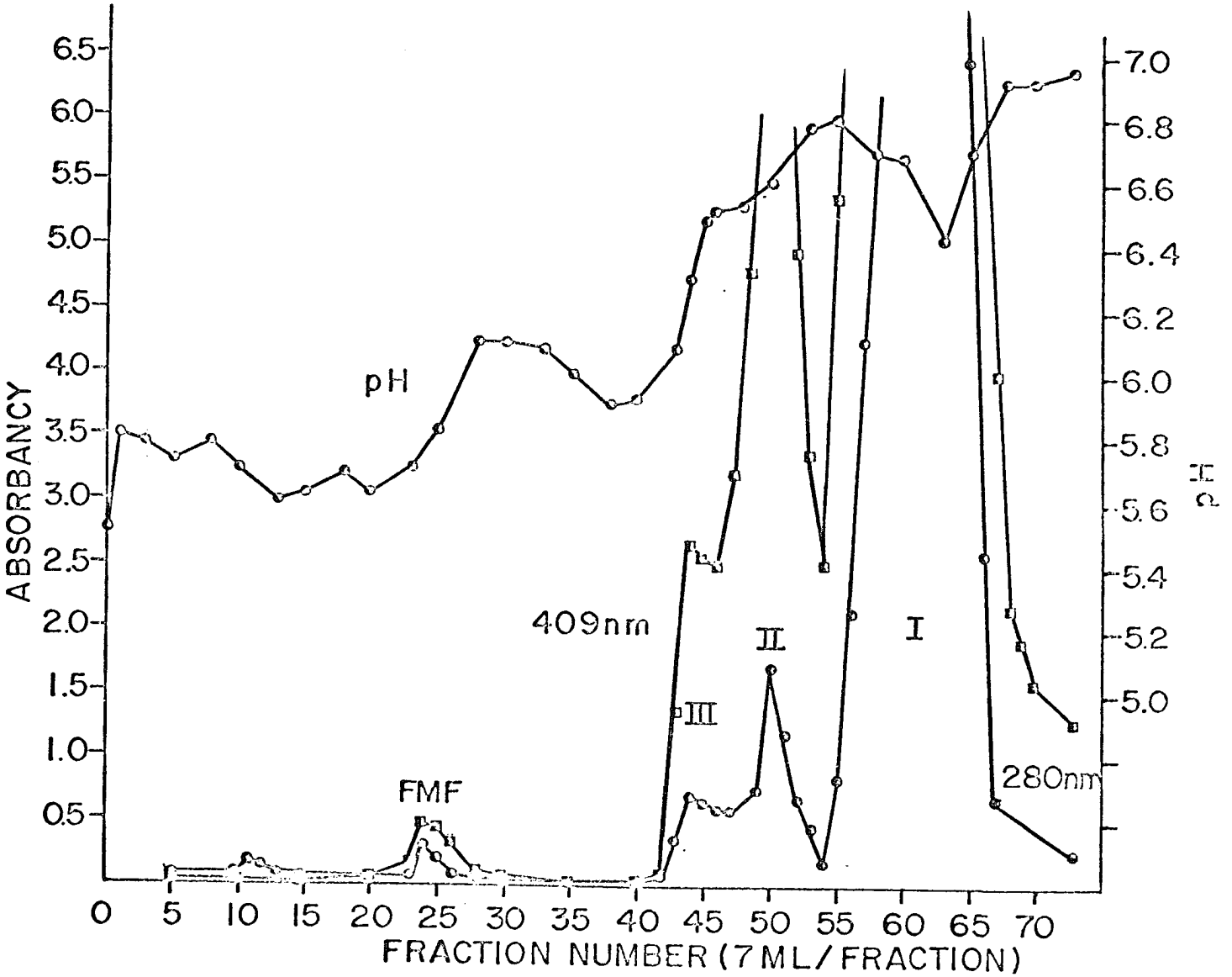
Table 1 also shows that there are two exceptions where column capacity was not the main factor in determining the size of the FMF (compare columns H and I to B, and column D to E). In each instance the column of the higher pH had the larger FMF.

Fig. 5 shows the elution pattern of a column with a starting pH of 5.5. This elution curve differs in two ways from the pH 6.0 column shown in Fig. 2 and comparable in size. A very small FMF was eluted at pH 5.5, consisting of approximately 0.5% of the total protein eluted; the FMF from a pH 6.0 column of similar dimensions was approximately 4% of the total protein eluted. The lower starting pH seemed to shift the elution curve toward a higher percentage of more positively charged myoglobin, i.e. a larger amount of Mb I. Furthermore, chromatography at pH 5.5 resulted in the elution of four fractions.

Equilibration of the fast-moving fraction

It was observed that Quinn, Pearson, and Brunner (1964) obtained an elution curve at pH 6.9 which resembled most the curve at pH 6.0 shown in Fig. 1, though they used a much larger column for approximately the same amount of myoglobin. This, and the previously cited data, indicated that

Fig. 5. CM-Cellulose chromatogram of bovine metmyoglobin. Approximately 437 mg purified myoglobin in 0.01 M potassium phosphate buffer, pH 5.5, was applied to the column, 2.5 x 18 cm bed dimensions. Estimated amount of myoglobin eluted in each fraction: Mb I, approximately 94%; Mb II, 4.5%; Mb III, 1%; FMF, 0.5%


ABSORBANCY
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
pH
409nm
280nm
FMF
I
II
III
7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6
5.4
5.2
5.0
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
FRACTION NUMBER (7 ML/FRACTION)

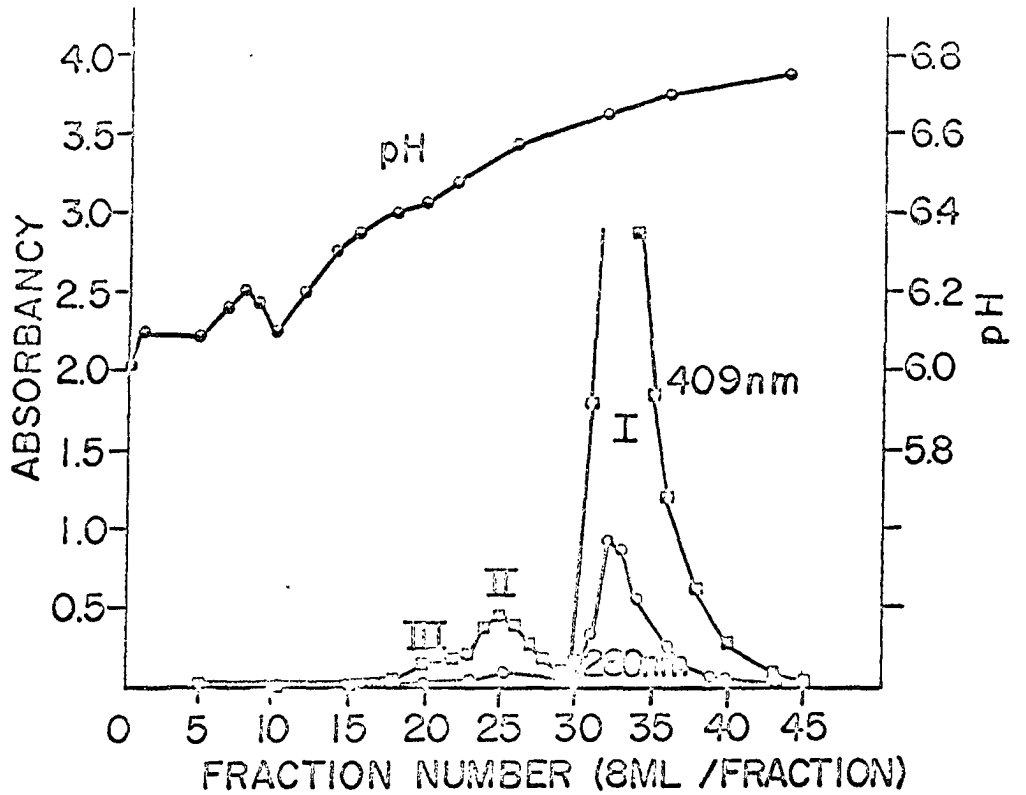
the equilibrating pH has as great an effect on the elution pattern as does column capacity.

To prove this point, two identical columns 1.5 x 25 cm were each equilibrated at a different pH and 22-23 mg of myoglobin was chromatographed on each. Figs. 6 and 7 show the elution curves for these columns. At pH 6.0 (Fig. 6) this small amount of myoglobin gave the same type of elution pattern seen in Fig. 4 where 300 mg of myoglobin was chromatographed on a 2.5 x 60 cm column. On both columns a FMF started out as a distinct band moving down the column far ahead of the rest of the myoglobin. When this FMF had migrated about half-way down the column, it became visibly less and less concentrated and seemed to be equilibrating with the column material. Finally, this FMF disappeared completely instead of emerging at an early point in the elution curve as would have been expected from its initial mobility. This altered the elution pattern considerably from those shown in Figs. 1 and 2. In the latter columns the FMF was eluted rapidly with little or no visible equilibration of the protein with the column material. Fig. 8 is a series of four photographs showing the formation of the fast-moving band and its eventual equilibration with the column material within the first half hour of starting the elution.

At pH 6.8 (Fig. 7), an identical column to the pH 6.0 column just described, the same amount of myoglobin separated into two bands when first placed on the column bed. Both bands migrated down the column at the same rate and were eluted in the sequence expected from their initial mobilities. Fig. 7 shows that a fairly large (7%) FMF was eluted at pH 6.8 but not at pH 6.0 (Fig. 6). The migration of the two bands on the

Fig. 6. CM-Cellulose chromatogram of bovine metmyoglobin. Approximately 23 mg of purified myoglobin in 0.01 M potassium phosphate buffer, pH 6.0, was placed on the column, 1.5 x 25 cm bed dimensions. Estimated amount of myoglobin eluted in each fraction: Mb I, approximately 92.5%; Mb II, 5%; Mb III, 2.5%

Fig. 7. CM-Cellulose chromatogram of bovine metmyoglobin. Approximately 22 mg of purified myoglobin in 0.01 M potassium phosphate buffer, pH 6.8, was placed on the column, 1.5 x 25 cm bed dimensions. Estimated amount of myoglobin eluted in each fraction: Mb I, approximately 93%; FMF, 7%


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ABSORBANCY
pH
6.8
6.6
6.4
6.2
6.0
5.8
pH
409nm
I
II
III
280nm
0 5 10 15 20 25 30 35 40 45
FRACTION NUMBER (8ML /FRACTION)

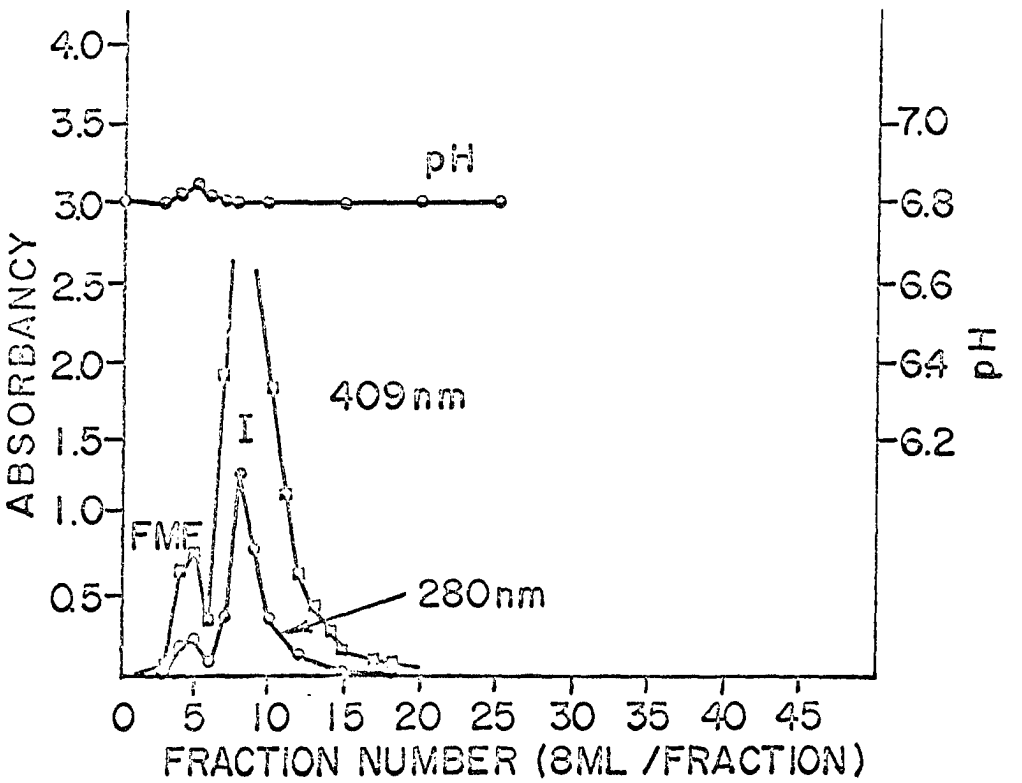
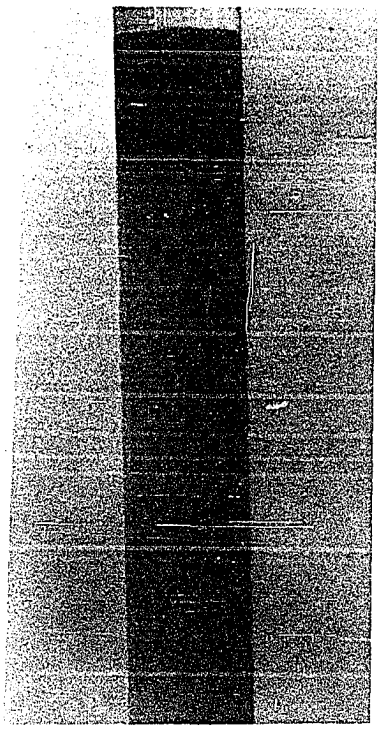
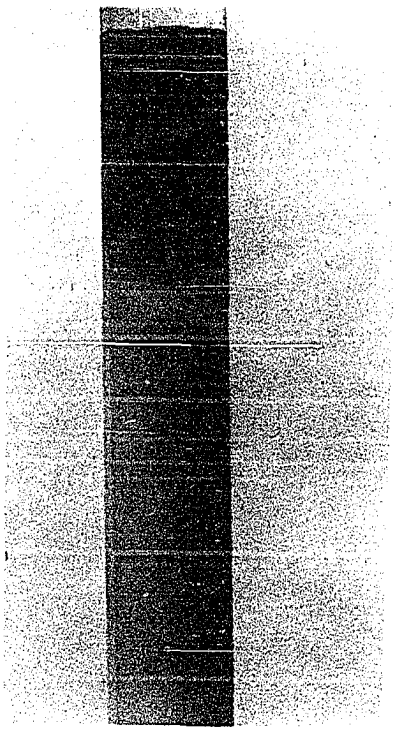
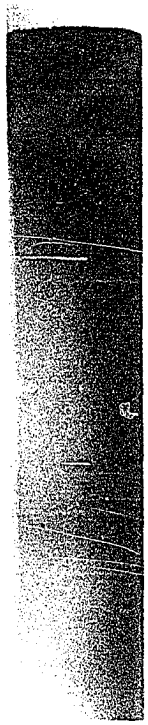

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
ABSORBANCY
pH
7.0
6.8
6.6
6.4
6.2
pH
409nm
I
FME
280nm
0 5 10 15 20 25 30 35 40 45
FRACTION NUMBER (8ML /FRACTION)

Fig. 8. Series of 4 photographs shown in sequence from left to right, top to bottom of page, showing the process of equilibration of the FMF with the column material, and its eventual disappearance as a discreet fraction. These photographs were taken from the pH 6.0 column shown in Fig. 6







pH 6.8 column, and their elution in the same sequence, is shown in three photographs in Fig. 9.

The relationship of column size, and amount of myoglobin chromatographed, to the size of the FMF was not entirely surprising. This could be explained on the basis of column capacity; i.e. the need for a minimum number of exchange groups to effect the complete separation of a given amount of protein on a column. An explanation for the pH-dependent change in size of the FMF was not as readily available.

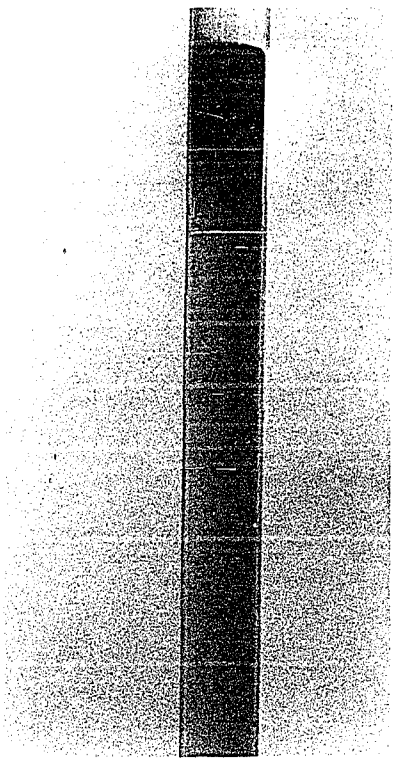
Change in pH of effluent from columns

As the FMF was eluted (Figs. 1, 2, 5) a concomittant rise in pH was observed. This rapid rise and decline in pH seemed to take place before the pH gradient on the column took effect. An attempt was made to correlate the change in hydrogen ion concentration to the amount of myoglobin eluted in the FMF. There seemed to be no correlation between the two. Because the myoglobin was equilibrated with the starting buffer, the possibility was eliminated that the rise in pH was due to poor buffering capacity for the amount of protein involved.

A CM-Sephadex column was run at pH 6.0 (Fig. 10) to see if the same elution pattern could be obtained with a column material other than CM-Cellulose. The elution curve obtained with CM-Sephadex at pH 6.0 (Fig. 10) differed markedly from the elution curve of a similar pH 6.0 CM-Cellulose column (Fig. 2), eluted under the same experimental conditions. In fact, the elution curve of the CM-Sephadex column at pH 6.0 resembled most the elution curve of a CM-Cellulose column at pH 5.5 (Fig. 5).

Samples for all three columns (Figs. 3, 5, 10) were prepared in the

Fig. 9. Series of 3 photographs shown in sequence from left to right, top to bottom of the page, showing the elution of the FMF and Mb I from a pH 6.8 column. These photographs were taken from a pH 6.8 column of the type shown in Fig. 7



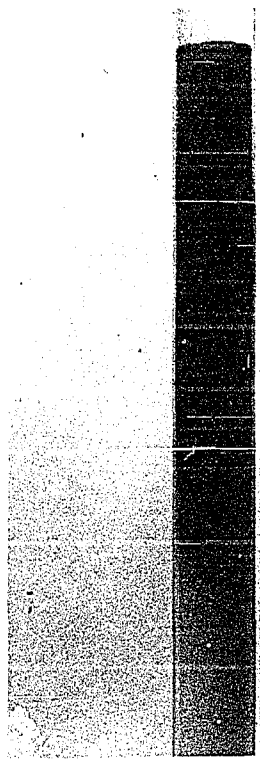
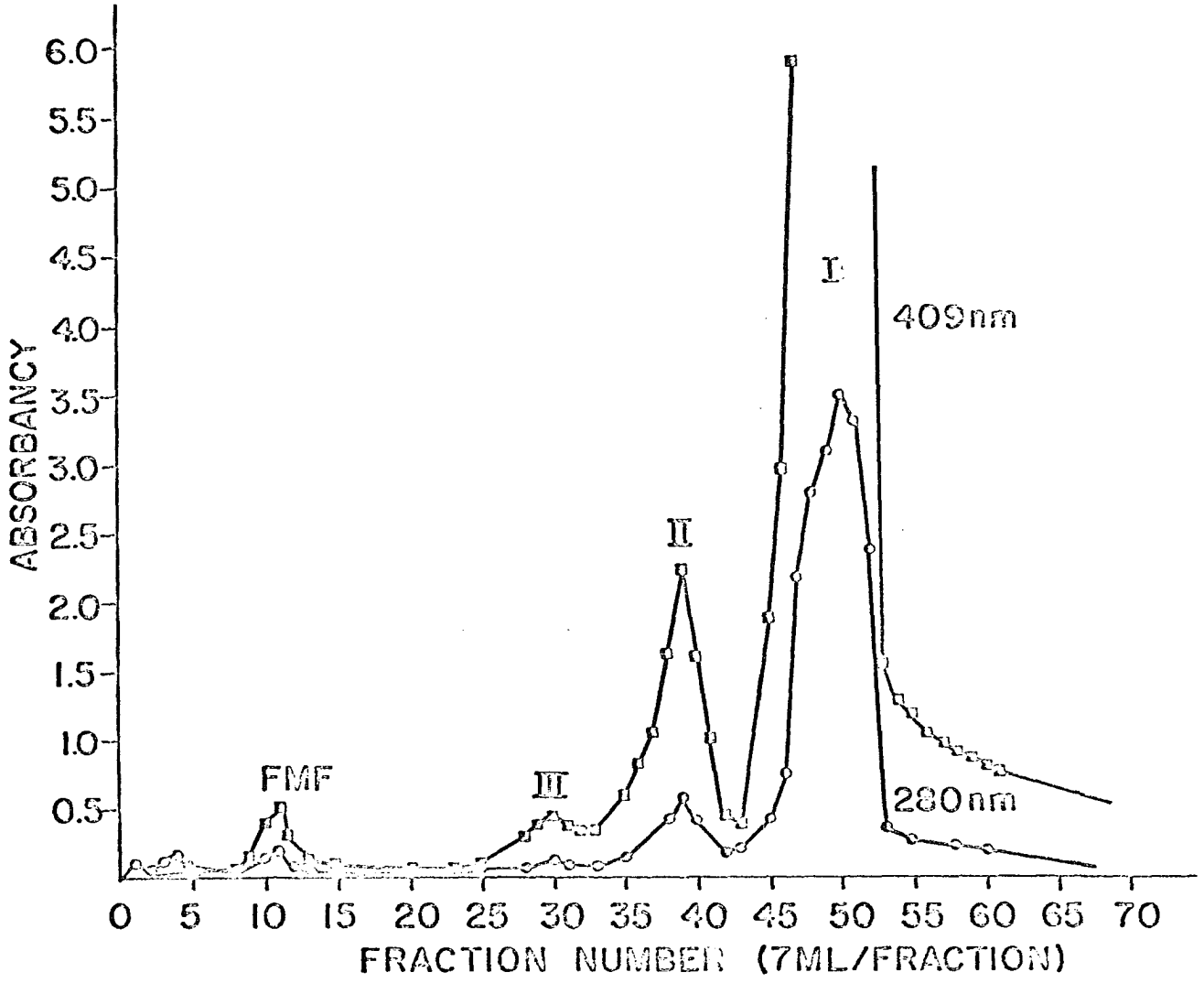


Fig. 10. Chromatogram from CM-Sephadex column equilibrated at pH 6.0. Approximately 155 mg purified myoglobin was placed on the column, 1.5 x 23 cm bed dimensions. Estimated amoung of myoglobin eluted in each fraction: Mb I, approximately 78.8%; Mb II, 13.7%; Mb III, 5%; FMF, 2.5%


ABSORBANCY
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
FMF
III
II
I
409nm
280nm
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70
FRACTION NUMBER (7ML/FRACTION)

same way but the elution curves still differed. Therefore, it seemed likely that there might be an interaction between the eluting buffer and the charged exchange groups on the column material. In order to examine this possibility, two CM-Cellulose columns were run without myoglobin. One column was equilibrated at pH 5.6, the other at pH 6.0. In each instance the same buffer gradient was passed through the column as when eluting myoglobin. The effluent was collected in the same manner as fractions from a regular column and the pH was then measured on each fraction collected.

Figs. 11 and 12 show the pH curves obtained for these two columns. Comparing these pH curves to myoglobin elution curves from similar columns, Figs. 2 and 5, it becomes evident that the fast-moving myoglobin fraction, in each instance, is eluted in the region of the pH curve where little change in pH is detected in the effluent, i.e. in the first 180 mls at pH 6.0 and the first 240 mls at pH 5.6. The pH of the gradient buffer mixture was checked after 280 mls had passed through the pH 5.6 column, just where the curve changed abruptly. The buffer mixture entering the column, at this point, had a pH of 6.7 but the effluent leaving the column had a pH of 5.6. The change in slope of the pH 5.6 curve occurs at 280 mls and is much more abrupt than the change in the pH 6.0 curve which occurs at 180 mls. The change in slope of these pH curves is followed by a fairly linear rise in pH from 6.6 on.

In order to maintain the pH of the effluent at the equilibrating pH after the gradient is started, the column material or the exchange groups have to contribute the needed hydrogen ion to the buffer gradient as the pH of the buffer mixture rises. This indicates some interaction between

Fig. 11. pH curve obtained from CM-Cellulose column, 2.5 x 18 cm, and equilibrated with 0.01 M potassium phosphate buffer, pH 5.6. The buffer gradient was formed and passed through the column as described under Methods, except that no myoglobin was placed on the column bed

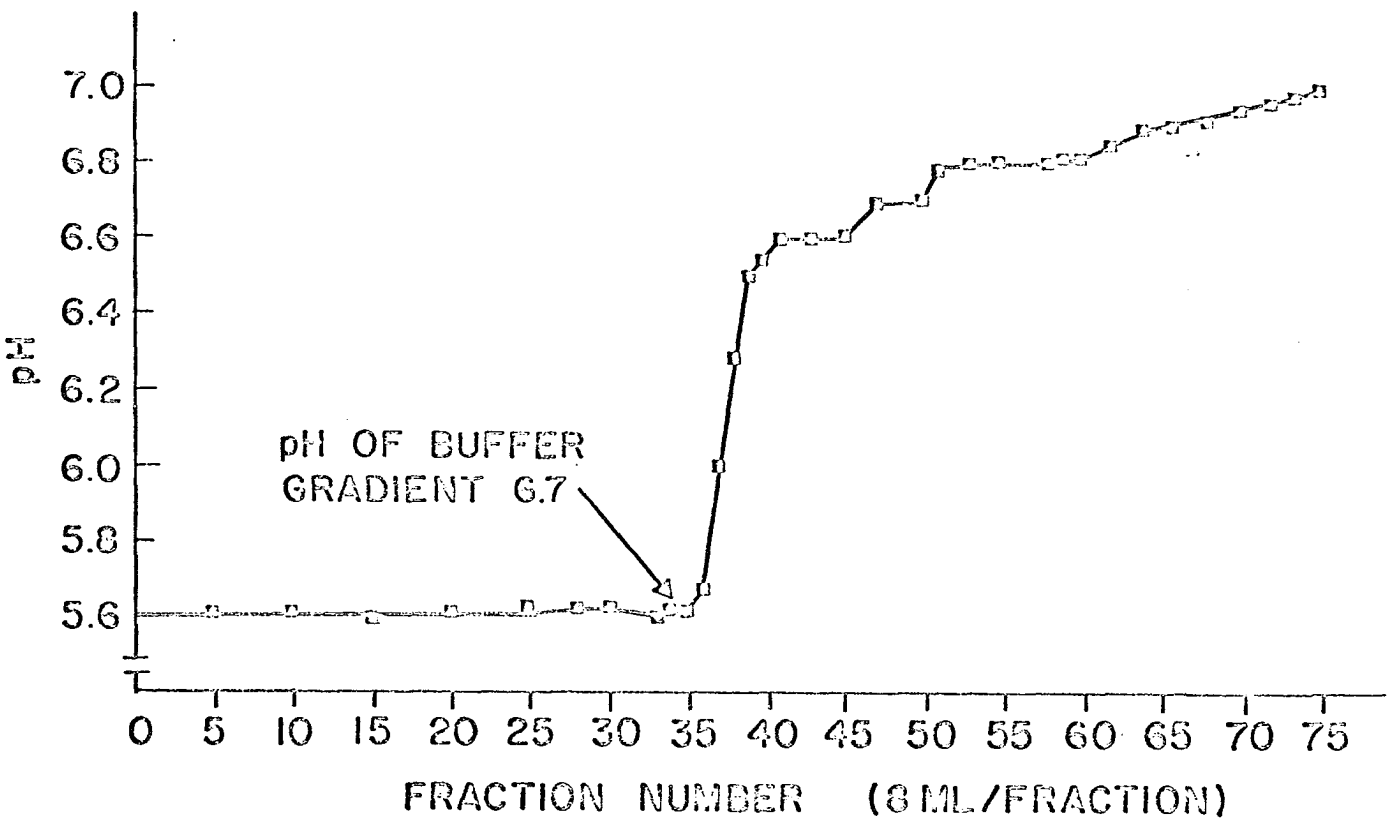
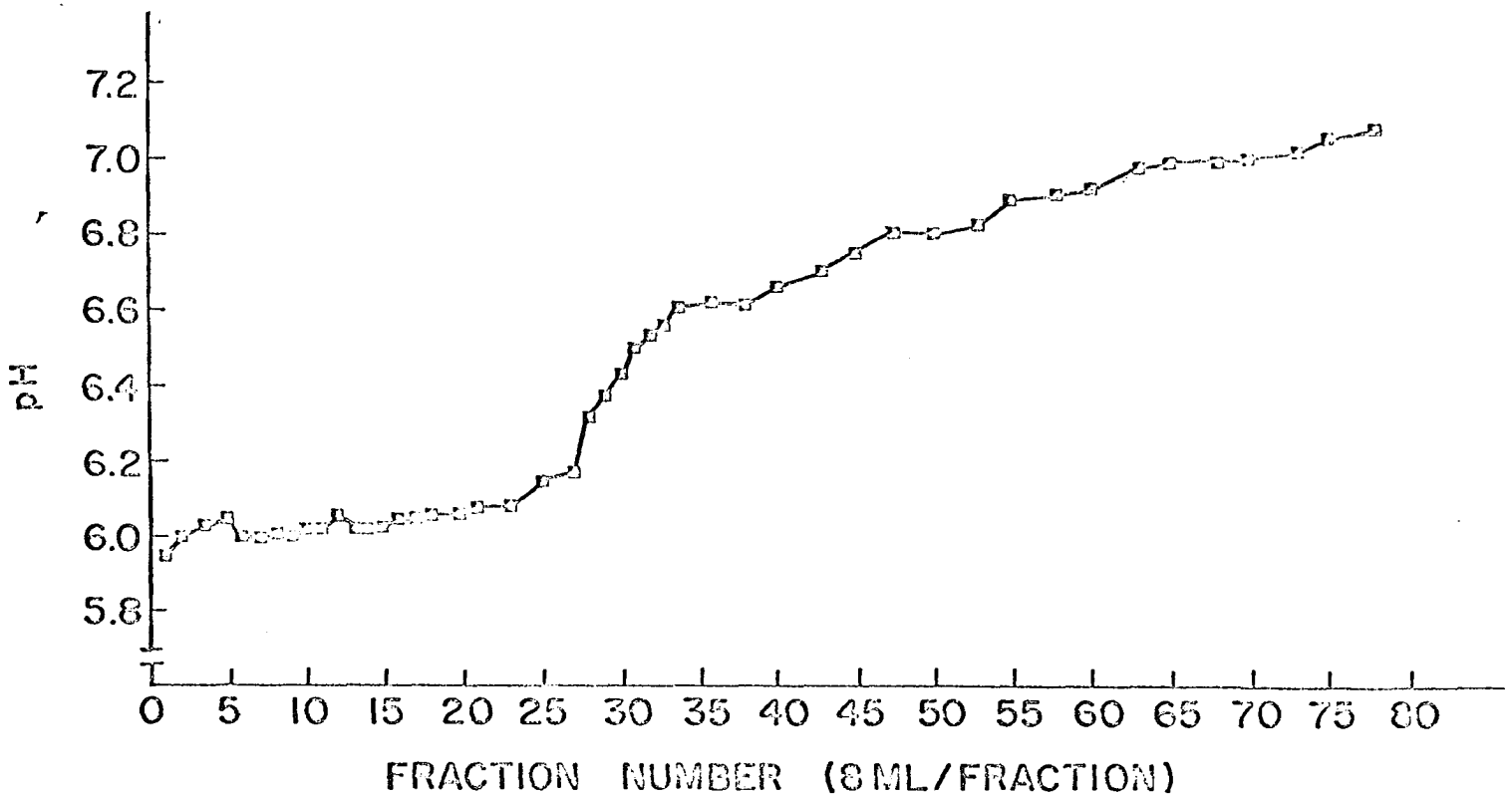

7.0
6.8
6.6
6.4
6.2
6.0
5.8
5.6
pH
pH OF BUFFER
GRADIENT 6.7
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75
FRACTION NUMBER (8 ML/FRACTION)

Fig. 12. pH curve obtained from a CM-Cellulose column, 2.5 x 18 cm, and equilibrated with 0.01 M potassium phosphate buffer, pH 6.0. The buffer gradient was formed and passed through the column as described under Methods, except that no myoglobin was placed on the column bed


pH
7.2
7.0
6.8
6.6
6.4
6.2
6.0
5.8
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
FRACTION NUMBER (8 ML/FRACTION)

the column material and the buffer.

The same abrupt change in pH was observed on the pH 5.9 and 5.5 columns where myoglobin had been chromatographed (Figs. 2 and 5). This abrupt change in pH occurs at the same volume as on the pH curves, and is of the same magnitude. However, Figs. 2 and 5 show that elution of the FMF is accompanied by a rise in pH that is not related to the above phenomenon, and which occurs in the region equivalent to that on the pH curves where no change in pH is observed; namely, between 150 and 170 mls on the pH 5.5 column (Fig. 5), and 90-120 mls on the pH 5.9 column (Fig. 2). Apparently the change in pH that seems to accompany the elution of the FMF is not necessarily due to an interaction of the column material and the buffer, but might be due to the interaction of the protein and the column material. Reasons for the elution pattern obtained on CM-Sephadex are not clear and may have to do with column capacity. However, the problem was not pursued further.

The rise in pH accompanying elution of the FMF is not as marked on CM-Cellulose columns with increased capacity (see Figs. 4, 6, 7). Figs. 13, 14 and 15, presented in the next section, indicate that a fairly linear pH gradient is obtained upon rechromatography of the FMF, and the pooled Mb I and II fractions; and the initial rise in pH is small. In these instances, too, a small amount of protein was placed on the column.

Clayton and Bushuk (1966) showed a similar variability in pH on CM-Cellulose columns. They showed a greater variability in pH with decreasing buffering capacity of the eluant. They also showed that, with decreasing buffering capacity, a higher ionic strength is required to elute a given protein.

Clayton and Bushuk (1966) suggested that the adsorption process on CM-Cellulose involves displacement of sodium ions from the cellulose by positively charged basic groups on the protein. They suggest that this might cause a slight rise in pH since sodium ions are more basic than any basic protein groups. Also, they suggest that the reverse occurs when sodium ions in solution displace relatively weak basic positively charged protein groups from the cellulose.

The same reasoning might apply to the observed rise in pH that seems to occur when the FMF is eluted. Further support for the idea of the displacement of more basic ions by less basic ions lies in the fact that the magnitude of the pH change is not as great when less protein is placed on the column. Furthermore, a slight rise in pH is observed even when no FMF is eluted (see Figs. 4 and 6), indicating that the change in pH occurs as the myoglobin is placed on the column, and not as it is eluted.

The rise in pH of the eluant may cause some of the protein to become more negatively charged. This, in turn, would account for the rapid elution of the FMF at high pH's where the protein is also more negatively charged to begin with. At pH 5.5 the protein is more positively charged than at pH 6.0, so that, despite the change in pH, less myoglobin is eluted as a FMF.

However, the elution of a FMF, or its re-equilibration with the column material under certain conditions, may not be related to the observed rise in pH. Instead, it is possible that there is an initial change in ionic strength as myoglobin displaces potassium ions on the exchanger. Such an increase in ionic strength would cause some of the myoglobin to move down the column rapidly. However, if the column is long

enough (Fig. 4), the potassium ions are diluted out and the myoglobin is retained on the column gradually. In the same way, a short column with very little myoglobin on it (Fig. 6) would permit equilibration of the FMF by virtue of the fact that fewer potassium ions would have been displaced; consequently these ions are still diluted out even though the column is short.

There is no strong evidence to indicate whether the equilibration of the FMF is due to a change in ionic strength or whether it is in some way related to the observed changes in pH. It is conceivable that neither of these factors is involved in the ultimate explanation of this phenomenon.

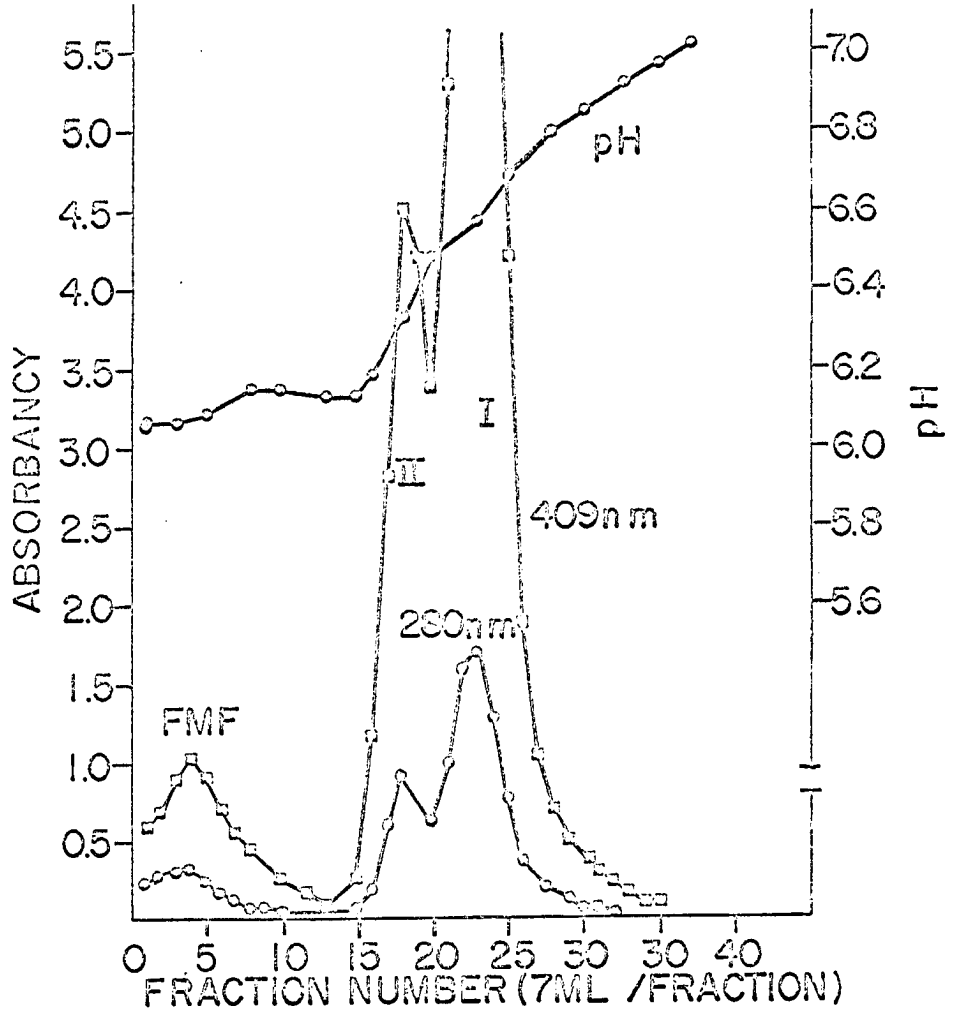
The possibility of artifactual fractions due to inadequate pH control can be eliminated on the basis of evidence to be presented later; namely, electrophoretic heterogeneity of all column fractions, and heterogeneity of the FMF on rechromatography, even when a fairly linear pH gradient is obtained.

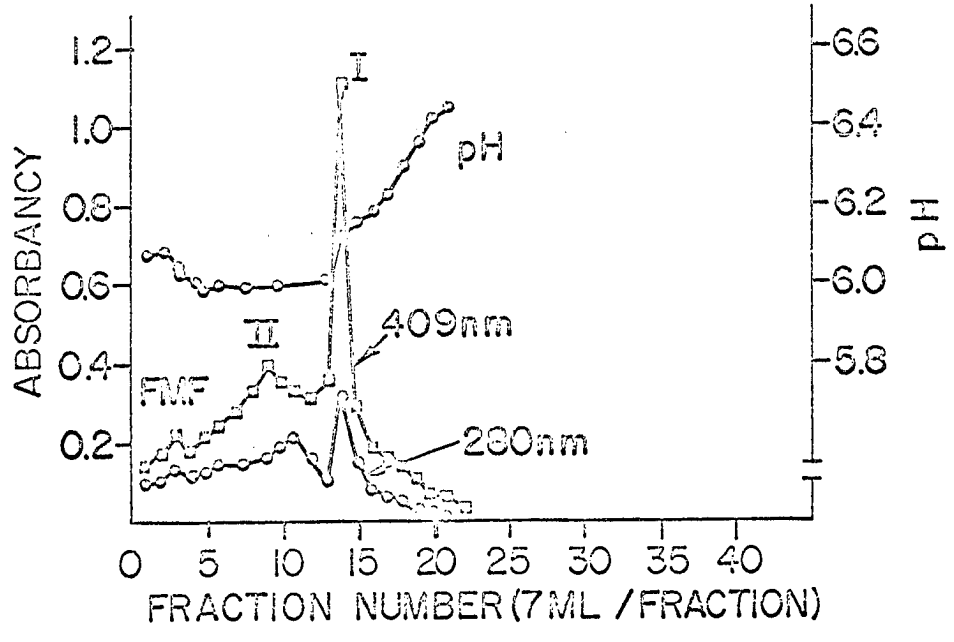
Rechromatography of the fast-moving fraction

In an effort to further purify the FMF eluted from a column of the type shown in Fig. 1, the pooled fraction was re-equilibrated against 0.01 M potassium phosphate buffer and rechromatographed under the same conditions. The elution curve obtained on rechromatography is shown in Fig. 13. Instead of obtaining a single peak, as might be expected from a homogeneous fraction, the FMF was converted into the three components seen in Fig. 2. The elution curve in Fig. 13 indicates that most of the myoglobin in the FMF (about 95% of it) was converted into Mb I and II, the most positively charged myoglobin components. In fact, rechromatography of the FMF

Fig. 13. Chromatogram of the rechromatography of the FMF at pH 6.0 on a CM-Cellulose column 2.5 x 5 cm (24.5 cc). This resulted in the conversion of approximately 95% of the protein into Mb I and II

Fig. 14. Chromatogram of the rechromatography of the FMF from Fig. 13. A CM-Cellulose column 0.8 x 7.5 (3.8 cc), pH 6.0 was used. Rechromatography resulted in the conversion of approximately 92% of the FMF into Mb I and II


ABSORBANCY
pH
409nm
280nm
FMF
I
II
FRACTION NUMBER (7ML /FRACTION)


ABSORBANCY
pH
409nm
280nm
FMF
I
II
FRACTION NUMBER (7 ML / FRACTION)

resulted in the same elution pattern obtained for purified metmyoglobin.

The FMF from rechromatography, i.e. the FMF from Fig. 13, was again re-equilibrated and rechromatographed. Due to the low concentration of protein a small column (0.8 x 7.5 cm) was used. This changed the point of emergence of the fractions. However, Fig. 14 shows that, once more, about 92% of the FMF was converted predominantly into Mb I and Mb II.

Mb I and II from the rechromatographed FMF in Fig. 13 were pooled and rechromatographed. The elution curve for these fractions (Fig. 15) shows that there is a very slight conversion if any, to the FMF. However, the more positive components still predominate at pH 6.0.

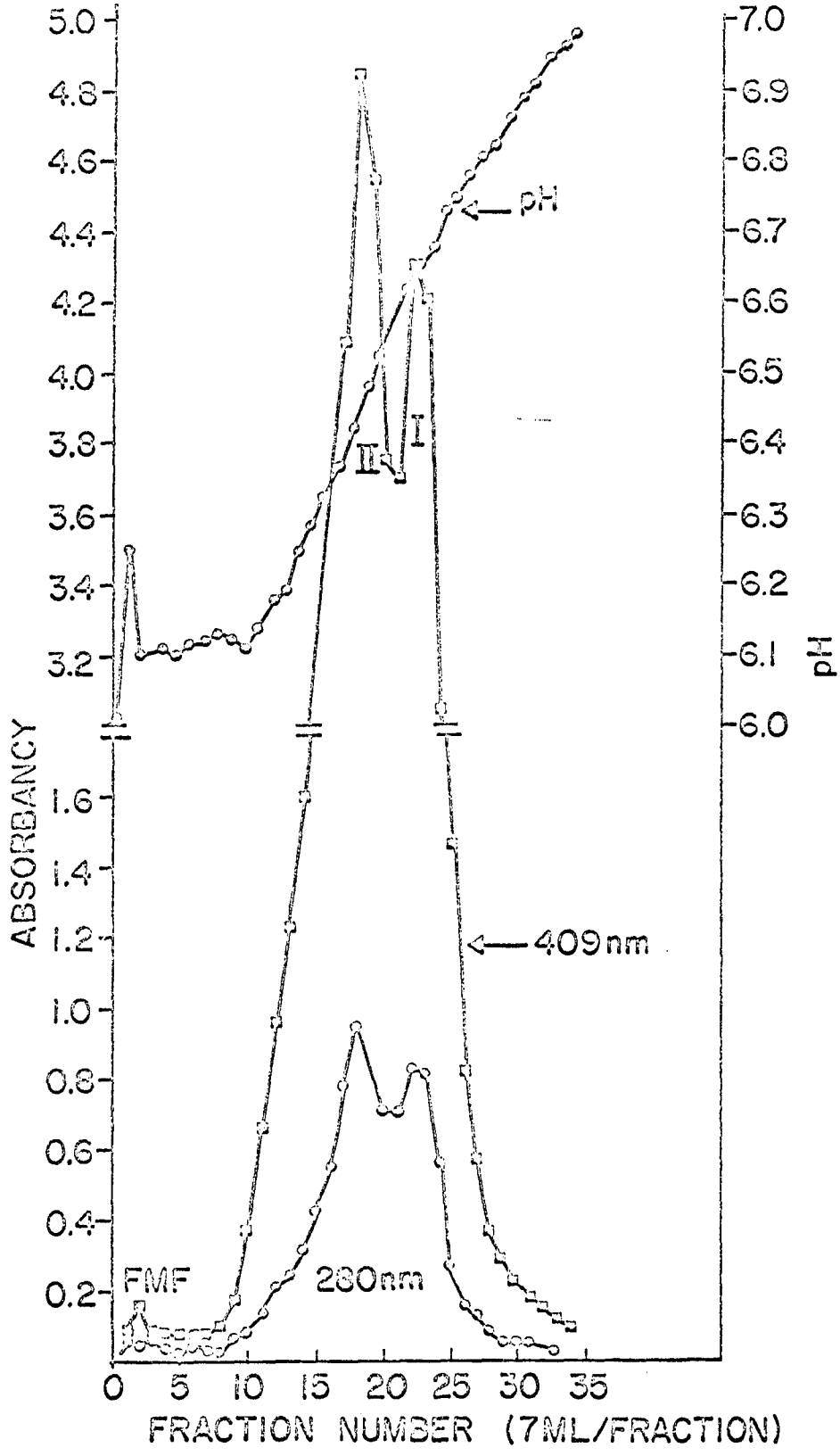
This recurring but consistent change in the proportions of fractions obtained from rechromatography of the FMF indicated a possible conversion of this component into all the others on CM-Cellulose columns. Rechromatography of Mb I and II indicates that these components are not as much subject to such changes.

These results indicated that the FMF consisted either of a mixture of all myoglobin components to begin with, or that the FMF underwent a change during chromatography that resulted in this mixture.

Further evidence for changes in CM-Cellulose fractions is presented in the section on electrophoresis.

The 409/280 nm ratios of the rechromatographed fractions were lower than the ratios of the original sample. This indicated the possibility of a low 409 nm, or a high 280 nm reading due to a conformational change in the protein upon dilution. This point was checked by taking difference spectra at both wavelengths. Purified myoglobin was placed in a 0.2 cm cell, and a 5 cm cell with buffer was lined up behind it in the cell-

Fig. 15. Chromatogram of the rechromatography of the pooled fractions, Mb I and II, from Figure 13. Chromatography was carried out at pH 6.0 on a CM-Cellulose column 2.5 x 4.5 cm (22.6 cc)


5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
7.0
6.9
6.8
6.7
6.6
6.5
6.4
6.3
6.2
6.1
6.0
pH
ABSORBANCY
II
I
409nm
FMF
280nm
0 5 10 15 20 25 30 35
FRACTION NUMBER (7ML/FRACTION)

holder for the Beckman DK-2A recording spectrophotometer. Next to these were lined up a 0.2 cm cell containing buffer behind which was placed a 5 cm cell containing a 1:25 dilution of the same myoglobin sample. Difference spectra recorded at both wavelengths showed no dilution effect.

Dilution of the protein apparently was not the reason for obtaining the low 409/280 nm ratios on rechromatography. The low ratios might be due to a high 280 nm reading caused by some light-scattering material picked up from the column. The problem was not pursued further.

Polyacrylamide Gel Vertical Electrophoresis

Fig. 16 D (identical to Fig. 3 D) shows the electrophoretic pattern of myoglobin purified by ammonium sulfate fractionation. Fig. 16 A, B, C (identical to Fig. 3 A, B, C) are the electrophoretic patterns obtained from Mb I, II, and the FMF from a pH 6.0 CM-Cellulose column, 2.5 x 17.5 cm (Fig. 2). The discontinuous system of buffers (Poulik, 1957) was used; pH of the gel and sample was 8.6, the circulating buffer had a pH of 7.9. As can be seen, none of these fractions was homogeneous.

At this high pH, on the alkaline side of the isoelectric point of myoglobin, purified metmyoglobin (Fig. 16 D) was resolved into four electrophoretic components, e-1, e-2, e-3 and e-4 in order of decreasing concentration. Occasionally, a fifth and sometimes a sixth, electrophoretic component could be seen.

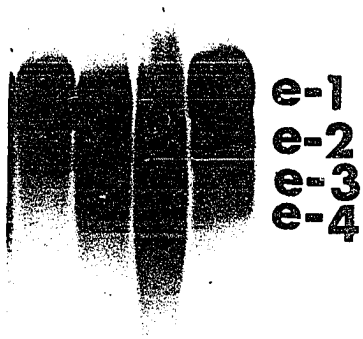
CM-Cellulose Mb I (Fig. 16 A), the major chromatographic component, was resolved into three electrophoretic components, e-1, e-2 and some e-3, with e-1, the most positively charged component, predominating. The fourth component seen in purified myoglobin, e-4, was either missing or present

Fig. 16. Polyacrylamide gel electrophoresis of purified myoglobin from a pH 5.9 CM-Cellulose column (2.5 x 17.5 cm). Electrophoresis of these samples was done in an 8% gel by the vertical gel technique, using Poulik's discontinuous system of buffers (pH 8.6). Origin of the gel is at the top; samples are migrating downward towards the positive pole. Samples are, from left to right:
A = Mb I
B = Mb II
C = FMF
D = Purified MetMb

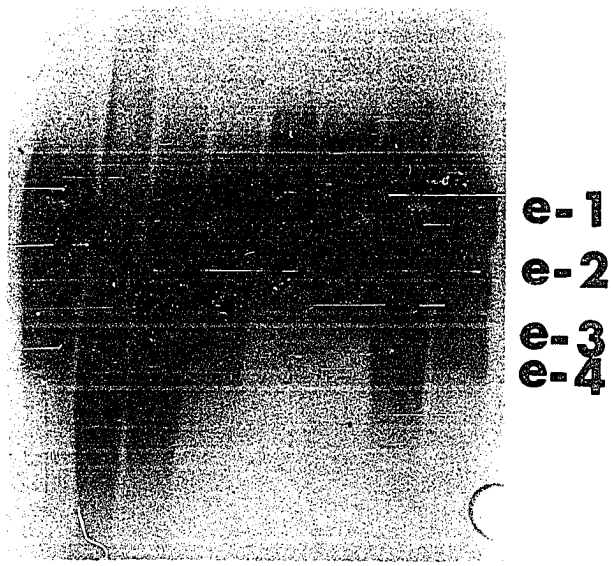
Fig. 17. Polyacrylamide gel electrophoresis carried out under the same conditions described in Fig. 16. The samples are, from left to right:
A = purified metMb
B = FMF from pH 5.5 column (Fig. 5)
C = Mb III from pH 5.5 column (Fig. 5)
D = Mb II from pH 5.5 column (Fig. 5)
E = Mb I from pH 5.5 column (Fig. 5)
F = Mb I from pH 6.0 column (2.5 x 5 cm)
G = FMF from pH 6.0 column (2.5 x 5 cm)
H = Purified metMb

Fig. 18. Polyacrylamide gel electrophoresis carried out as in Fig. 16. The samples are, from left to right:
A = Mb II from pH 6.0 column (Fig. 6)
B = FMF from pH 6.8 column (Fig. 7)
C = Mb I from pH 6.8 column (Fig. 7)
D = Mb I from pH 6.0 column (Fig. 6)
E = Mb I from pH 6.0 column (Fig. 4)
F = Purified metMb

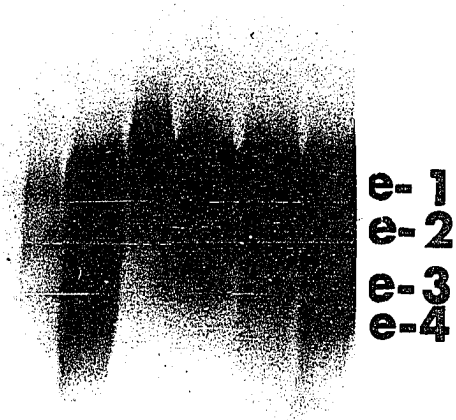
Fig. 19. Polyacrylamide gel electrophoresis carried out as in Fig. 16 except that EDTA was added to all buffers. Samples are, from left to right:
A = Mb I from pH 5.5 column (Fig. 5)
B = Mb II from pH 5.5 column (Fig. 5)
C = purified metMb held for one month or more in solution
D = Purified metMb



A B C D



A B C D E F G H



A B C D E F

A B C D

in too low a concentration to be detected. Mb II (Fig. 16 B) consisted of the same three electrophoretic components as Mb I but had a little less e-1 and more e-2 and e-3 than, Mb I. By contrast, the FMF (Fig. 16 C) consisted of very little e-1, more e-2 and e-3, and 3-4 which streaked far ahead of the e-4 component in purified myoglobin. This streaking, or lack of a discreet e-4 band, was also seen in the electrophoresis of the FMF in barbital buffer, pH 8.6. This CM-Cellulose fraction (FMF) consistently contained some myoglobin with a greater negative charge at a high pH than the e-4 component of the purified protein. Streaking was also seen in Mb II (Fig. 16 B) though to a lesser extent than in the FMF.

Similar streaking was observed on electrophoresis at pH's above 8.0 of the FMF obtained from columns equilibrated at different pH values (Fig. 17 B, C, G and Fig. 18 B), namely, pH 5.5, short (2.5 x 5 cm) pH 6.0, and pH 6.8 columns. When barbital buffer, pH 8.6 was used, one less electrophoretic component was observed in purified myoglobin and CM-Cellulose fractions than with the discontinuous system of buffers. However, the same relative proportions of slow to fast electrophoretic components were observed with both buffers.

A comparison of Fig. 16 C with Fig. 17 G further reveals that the FMF from a short (2.5 x 5 cm) pH 6.0 column contains more e-1 than the FMF from a longer (2.5 x 18 cm) pH 6.0 column. This was interpreted as additional confirmation that a better separation is obtained on columns of greater capacity, with a consequent elution of a smaller FMF containing less e-1. However, the heterogeneity of CM-Cellulose fractions is evidently not due to inadequate column capacity. The 2.5 x 60 cm column (Fig. 4) was not over-loaded by any standards; neither was the 1.5 x 25 cm, pH 6.0, column

shown in Fig. 6. Yet, unexpectedly, the major fraction from each of these columns was heterogeneous on electrophoresis (Fig. 18 E and D).

Heterogeneity and metal-binding

The possibility that some of the fractions might be caused by the presence of metals was investigated. EDTA, 10^{-3} M, had no effect on the number of CM-Cellulose fractions eluted. On electrophoresis at a given pH, with and without EDTA in the buffers, the same electrophoretic components were obtained for purified myoglobin and fractions from CM-Cellulose columns (compare Fig. 17 D, E and H with Fig. 19 B, A and D).

Fig. 19 C and D both show electrophoretic patterns of purified myoglobin. Sample D was freshly prepared just prior to electrophoresis; sample C had been standing in solution for a month or more. The same electrophoretic components were obtained with both samples, indicating the stability of the electrophoretic components at a given pH.

Heterogeneity versus homogeneity with 2-mercaptoethanol

Wolfson et al. (1967) claimed to reduce the multiple bands in human myoglobin to one component by using 2-mercaptoethanol in the agar gel on electrophoresis. In view of this unexpected finding an attempt was made to reproduce these results.

When 2-mercaptoethanol was used in preparing the gel, the acrylamide did not polymerize. Apparently the 2-mercaptoethanol, acting as a reducing agent, was interfering with the catalytic action of ammonium persulfate in initiating the polymerization of the acrylamide. Electrophoresis was then performed under a hood by using 2-mercaptoethanol in the sample and circulating buffer but not in the gel.

Addition of 2-mercaptoethanol to metmyoglobin changed the typical met color to a deep red. Spectrophotometric analysis in the visible range (700 to 450 nm) revealed that the sample consisted mostly of oxymyoglobin but still had some metmyoglobin in it. Apparently 2-mercaptoethanol reduced the iron in the heme. Electrophoresis of this mixture showed a very small band of metmyoglobin moving much faster than the oxymyoglobin toward the positive pole. The red oxymyoglobin was seen to separate into the same components normally seen with metmyoglobin in the absence of 2-mercaptoethanol.

However, upon staining with the benzidine stain, some minor bands, though initially visible without staining, gradually disappeared. Repeated changing of the staining material had no effect on the staining of the minor components. Only the major band remained visible. Apparently the 2-mercaptoethanol was interfering in some way with the staining procedure but was not eliminating the microheterogeneity of myoglobin as suggested by Wolfson et al. (1967). It was noted that these investigators used the benzidine stain to show the elimination of all but one electrophoretic band in myoglobin by 2-mercaptoethanol.

Heterogeneity of cyanmetmyoglobin

Perkoff (1962), working with human myoglobin, had suggested that, at high pH's on DEAE-Cellulose, some myoglobin fractions are due to the acid-alkaline froms of the protein. Quinn and Pearson (1964) agreed with Perkoff. They found that some of the fractions of bovine myoglobin are eliminated on DEAE-Cellulose columns when cyanmetmyoglobin is used. However, Atassi (1964) obtained more fractions on CM-Cellulose than any other

investigator when he used the cyanide derivative of sperm whale metmyoglobin.

Fig. 20 D shows the same four electrophoretic components in the cyanide derivative of purified bovine myoglobin as were seen in Figs. 16 D, 17 H, 18F and 19 D for purified myoglobin. The cyanide derivatives of Mb I, II and III from a pH 5.5 column (Fig. 20 A, B, C) also show the same electrophoretic components as Mb I, II and III in the metmyoglobin form (Fig. 17 C, D, E). These results agree with the findings of Hardman et al. (1966) for sperm whale myoglobin. In neither instance was there evidence to support Perkoff's contention that all or some of the microheterogeneity of myoglobin was due to the separation of the acid-alkaline forms of the protein.

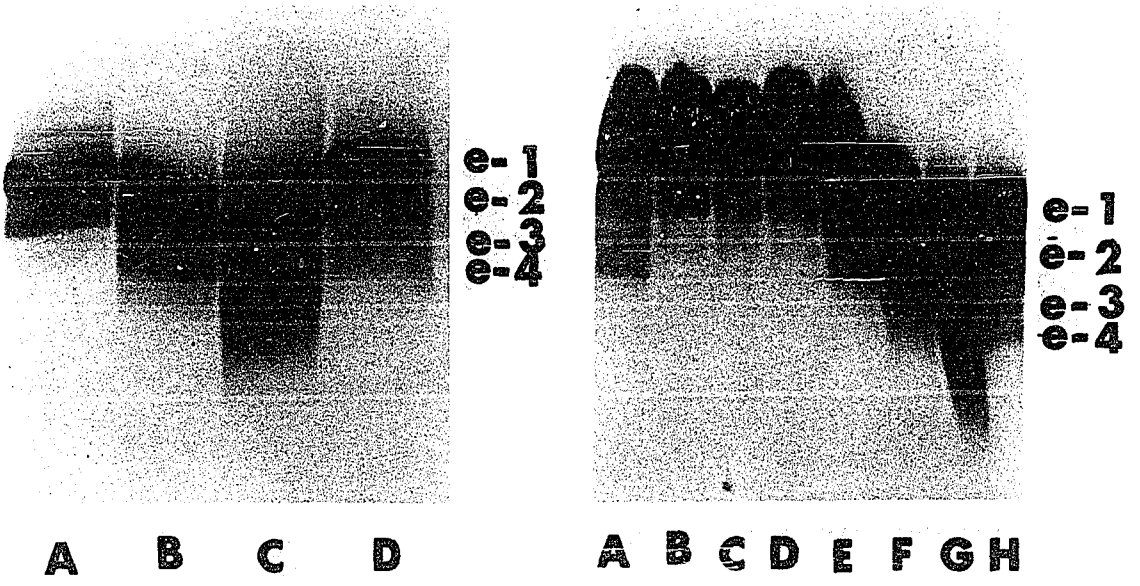
Heterogeneity and the equilibration of the fast-moving fraction

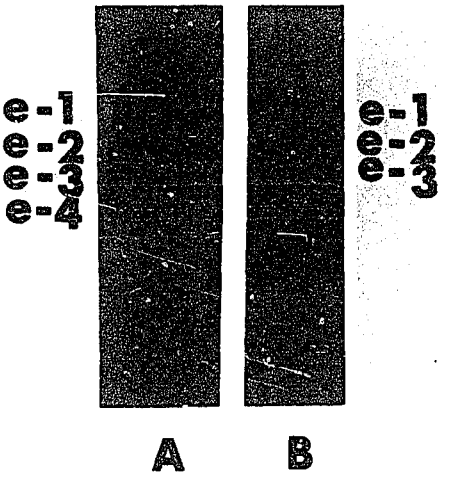
The FMF on CM-Cellulose columns has been shown to equilibrate with the column material under certain conditions (see the section on chromatography). On electrophoresis at pH 8.6 none of the CM-Cellulose fractions was found homogeneous. These two observations led to the speculation that the contamination of each fraction with the other might be occurring on the column due to the equilibration of the FMF with the column material. If the FMF is an artifact caused by poor initial adsorbtion of some of the protein due to an increase in ionic strength, it would be expected to contain a mixture of all the myoglobin components. After the FMF equilibrates with the column material, Mb I and II could, conceivably, become contaminated with some components from the FMF as they move down the column and pass over the area where it had re-adsorbed on

Fig. 20. Polyacrylamide gel electrophoresis of cyanmetmyoglobin in Poulik's discontinuous system of buffers (pH 8.6) with 0.01% KCN added. Origin of 5% gel is at top, samples are migrating downward toward the positive pole. Samples are, from left to right:
A = Mb I from pH 5.5 column (Fig. 5)
B = Mb II from pH 5.5 column (Fig. 5)
C = Mb III from pH 5.5 column (Fig. 5)
D = Purified metMb

Fig. 21. Polyacrylamide gel electrophoresis of myoglobin separated on a 2.5 x 22 cm column, pH 6.0, but not eluted. A 5% gel and Poulik's discontinuous system of buffers, pH 8.6, were used. Origin of gel is at the top; samples are migrating downward towards the positive pole. Samples are from left to right:
A = Purified metMb
B = Mb I
C = Section from column between Mb I and II
D = Mb II
E = Section from column between Mb II and III
F = Mb III
G = FMF
H = Purified metMb

Fig. 22. Polyacrylamide gel re-electrophoresis of myoglobin e-1 eluted from a 5% gel. Re-electrophoresis was carried out under the same conditions, using Poulik's discontinuous system of buffers (pH 8.6). Origin of the gel is at the top, samples are migrating downward towards the positive pole. Samples are, from left to right:
A = Purified metMb
B = Re-electrophoresis of major electrophoretic band, e-1


e-1
e-2
e-3
e-4
A B C D
e-1
e-2
e-3
e-4
A B C D E F G H


e-1
e-2
e-3
e-4
e-1
e-2
e-3
A B

the cellulose. The following experiment was designed to check the possibility of such overlapping of fractions during the elution.

About 500 mg myoglobin was placed on a column that was packed under 2-4 lbs pressure (final bed dimensions 2.5 x 22 cm). Conditions that result in the elution of a FMF were chosen. Potassium phosphate buffer at the equilibrating pH of 6.0 was passed over the column at a rate of 1 ml per minute. Myoglobin separated into four visible fractions on the column. When the FMF was within two inches of the bottom of the column, the whole column bed was pushed out in one piece by applying about 4 lbs pressure. The cellulose was sectioned off at the visible dividing lines of the fractions. It was observed that the inside column material at each division contained more myoglobin than the outer portion. These "overlapping" portions were cut off and treated as separate entities.

Each section of the column was then eluted with pH 6.8 potassium phosphate buffer-0.1 M NaCl. The filtrate was then dialyzed and lyophilized. Figs. 21 B and D show that Mb I and II from the column are still heterogeneous on electrophoresis though neither had migrated from its original position on the column. Each contained predominantly e-1, less e-2 and a trace of e-3. The FMF (Fig. 21 G) had the same electrophoretic components and in the same proportions observed previously for the eluted fraction.

These results indicated that contamination of Mb I and II with the other fractions does not result from the equilibration of the FMF on the column. However, only 3 fractions were eluted, though 4 fractions were visible on the column. It is, therefore, possible that intermediate fractions are contaminated one with the other due to equilibration of some

of the protein on the column. However, since Mb I did not move from its original position on the column but was still heterogeneous on electrophoresis, it seems likely that all myoglobin fractions from a column contain a mixture of the electrophoretic components which cannot be separated chromatographically.

Heterogeneity on re-electrophoresis at high pH's

To check the homogeneity of myoglobin on electrophoresis, component e-1 from two preparative gels, pH 8.6, was eluted with deionized water and lyophilized. Re-electrophoresis of e-1 on an analytical gel at the same pH showed that this band is not homogeneous (Fig. 22 B). It contained equal amounts of e-1, e-2, e-3. If e-4 was present it streaked ahead of the other components.

Because 30-40 mg had been used on the preparative run, it seemed possible that an imcomplete separation had been obtained. However, an incomplete separation would have resulted in more e-1 and less of the minor components. Since equal amounts of e-1, e-2 and e-3 were obtained on re-electrophoresis, it looked as though some of e-1 had been converted to the more negative components. This suggested a change in relative proportions, favoring the negative components at a high pH.

Two-dimensional electrophoresis at low and high pH's

Purified metmyoglobin was separated on electrophoresis using a 5% gel and the discontinuous buffer system of Poulik (1957). After separation of the sample into four components, a 5-6 mm wide strip was cut vertically. This 8-9 cm long gel strip was turned 90° and placed horizontally in a new 8% gel. The same buffer was used for electrophoresis in the second

dimension.

Fig. 23 shows that e-1 separated again into e-1, e-2 and e-3, though e-3 is not clearly visible in the photograph. If present, e-4 would be in too low a concentration to be detected by the staining technique. All four components in the original sample migrated diagonally in the second dimension, maintaining their same relative mobilities. This indicates that these components are not caused by aggregation of the protein. Due to their low concentration it is not apparent whether e-2, e-3 and e-4 were each converted into the other components during electrophoresis in the second dimension. However, this experiment did demonstrate that e-1 reverts to the original electrophoretic pattern of purified metmyoglobin at pH 8.6; i.e. the more negatively charged components were formed from the more positively charged e-1.

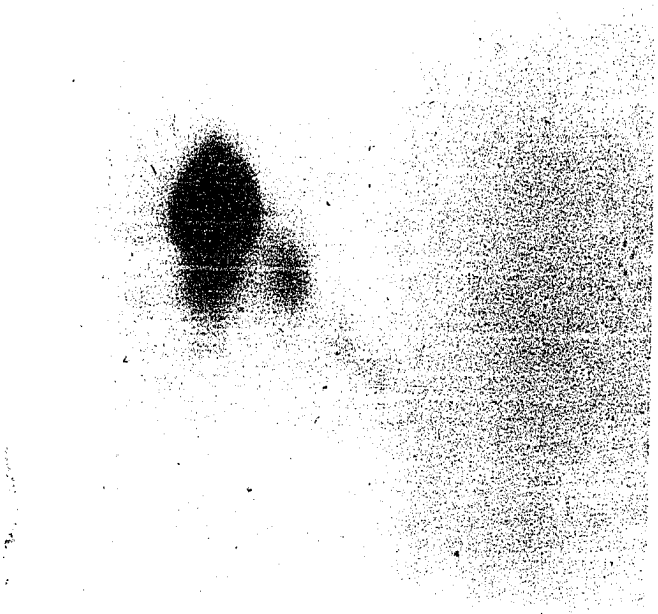
Fig. 24 shows the two-dimensional electrophoresis of purified metmyoglobin at pH 6.0. The major component, e-1, is again seen to revert to the original electrophoretic pattern of purified myoglobin at pH 6.0 (Fig. 25 A).

Electrophoresis at low pH's

The samples used for electrophoresis at a high pH (Fig. 16) are similar to those shown for electrophoresis at pH 6.0 (Fig. 25) and pH 5.2 (Fig. 26). Comparing purified metmyoglobin at pH 5.2 (Fig. 26 D), pH 6.0 (Fig. 25 A) and pH 8.6 (Fig. 16 D), it becomes evident that more of the minor components are formed at the high pH. At pH 6.0, e-1 and e-2 are seen, but e-3 is present in only trace amounts. At pH 5.2 only e-1 is a discrete component; e-2 is even less of a discreet band than at pH 6.0,

Fig. 23. Two-dimensional electrophoresis at pH 8.6 in polyacrylamide gels. A 5% gel was used in the first dimension, an 8% gel in the second dimension. Both dimensions were carried out in Poulik's discontinuous system of buffers. Origin of the gel is at the top, the sample is migrating downward towards the positive pole

Fig. 24. Two-dimensional polyacrylamide gel electrophoresis in potassium phosphate buffer, pH 6.0. A 5% gel was used in the first dimension, an 8% gel in the second dimension. Origin of the gel is the top, the sample is migrating downward towards the negative pole



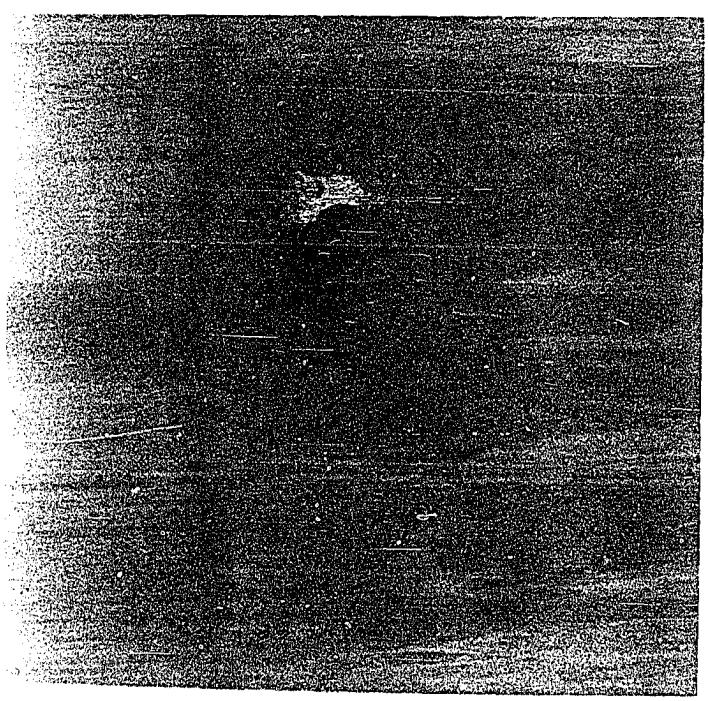
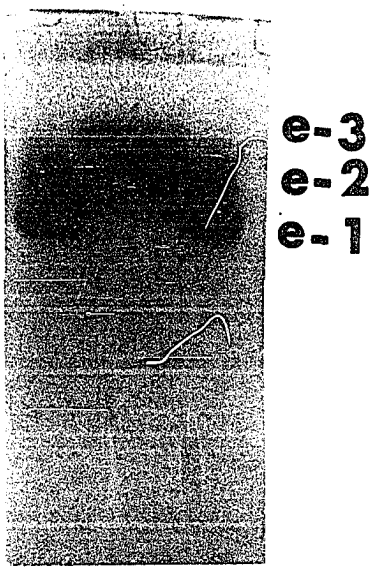
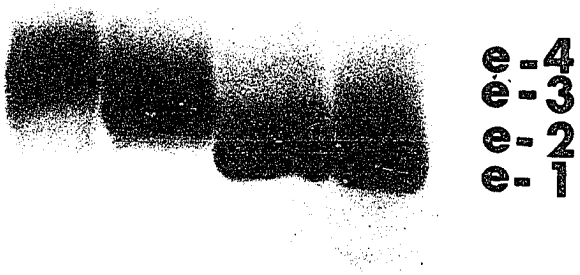


Fig. 25. Polyacrylamide gel electrophoresis in potassium phosphate buffer, pH 6.0. Origin of the 5% gel is at the top, samples are migrating downward towards the negative pole. Samples are, from left to right:
A = Purified metMb
B = FMF from a pH 6.0 column of the type shown in Fig. 2
C = Mb II from a pH 6.0 column of the type shown in Fig. 2
D = Mb I from a pH 6.0 column of the type shown in Fig. 2

Fig. 26. Polyacrylamide gel electrophoresis in sodium citrate buffer, pH 5.2. Origin of the 5% gel is at the top, samples are migrating downward towards the negative pole. Samples are, from left to right:
A = FMF from a pH 6.0 column of the type shown in Fig. 2
B = Mb II from a pH 6.0 column of the type shown in Fig. 2
C = Mb I from a pH 6.0 column of the type shown in Fig. 2
D = Purified metMb


e-3
e-2
e-1
A B C D


e-4
e-3
e-2
e-1
A B C D

and e-3 and e-4 are not seen as bands. At the high pH all four components are seen as well defined bands. The three fractions from a pH 5.9 column (Fig. 2), show similar pH-dependent shifts in concentration on electrophoresis. Specifically, Mb II, on electrophoresis at pH 5.2 (Fig. 26 B) and 6.0 (Fig. 25 C) contains distinctly more e-2 and less e-1 than the same fraction on pH 8.6 electrophoresis (Fig. 16 B).

Furthermore, on electrophoresis at pH 6.0 much of the FMF from CM-Cellulose columns ascended to the positive pole instead of migrating downward to the negative pole. This myoglobin was, apparently, still negatively charged at pH 6.0 and was lost in the circulating buffer. This indicated that one or more components in the FMF has an isoelectric point below pH 6.0. This was not observed on electrophoresis at pH 5.2.

A considerable difference in size of the FMF was shown on CM-Cellulose chromatography at pH 5.5 and 6.0. Conceivably at pH 6.0, the components with a low isoelectric point are still negatively charged and are easily eluted; whereas, at pH 5.5, some of these components are more positively charged and are thus adsorbed and retained on the column longer. This could result in the elution of a smaller FMF when myoglobin is chromatographed at pH 5.5. The components with an isoelectric point below 6.0, under the right circumstances, could account for the elution of a small FMF at pH 5.5, and the altered elution curve at that pH. However, the presence of such components alone does not necessarily explain the formation of a FMF at pH 6.0, nor the re-equilibration of it on the column, with its total disappearance as a discrete fraction under certain conditions.

DISCUSSION

The results presented in the previous section indicate that the microheterogeneity of myoglobin is not due to a protein-protein interaction of myoglobin with a non-heme contaminant; is not eliminated by 2-mercaptoethanol as suggested by Wolfson et al. (1967); is not due to the binding of metals by the protein (see experiment with EDTA); and is not due to the acid-alkaline forms of the protein as suggested in 1962 by Perkoff et al. (see experiment with cyanmetmyoglobin). The separation of acid-alkaline forms of myoglobin on a column is not a very likely explanation of microheterogeneity since a great deal of chromatography is carried out at pH values far below the pK of this reaction.

However, the results do show that it is possible to obtain from two to five chromatographic fractions by varying column capacity and/or equilibrating pH. It is also possible to alter the size and number of fractions eluted by changing the pH (compare Fig. 2 at pH 5.9 to Fig. 5 at pH 5.5, and Fig. 6 at pH 6.0 to Fig. 7 at pH 6.8). In each instance a larger FMF was eluted at the high pH.

Edmundson and Hirs (1962a) showed five elution curves from IRC-50 columns that demonstrated the sensitivity of the separations to small changes in pH. They showed that, at pH 5.92 the components moved relatively fast, with poor separation; at pH 5.82 they moved much more slowly with partial resolution; and at pH values below 5.82 they were strongly adsorbed to the resin.

The results presented here indicate that the behavior of myoglobin from different species, or any one species, on chromatography is not as

conflicting as might appear at first from perusal of the literature. At pH 6.8, Åkeson and Theorell (1960) resolved horse myoglobin into two broad fractions on CM-Cellulose. At the same pH, Quinn et al. (1964) resolved bovine myoglobin into three fractions. At pH 7.2, Stockwell resolved sperm whale myoglobin into two fractions on CM-Cellulose. In this study, bovine myoglobin was resolved into two fractions at pH 6.8 (Fig. 7), and two fractions at pH 6.0 on a 2.5 x 5 cm column (Fig. 1). At pH 6.0 on a 2.5 x 17.5 cm column (Fig. 2) bovine myoglobin was resolved into three fractions. This demonstrates the importance of starting pH as well as column capacity.

Hardman et al. (1966) resolved sperm whale myoglobin into 4 (or 5) chromatographic fractions on CM-Cellulose. Edmundson and Hirs (1962a) obtained similar resolution on IRC-50. Rumen (1959) resolved seal myoglobin into 5 chromatographic fractions. Fig. 4 in this study shows that, under certain conditions, bovine myoglobin can be resolved into 4 or 5 such graded fractions.

The only reported exception that is not completely explained on the basis of column capacity and/or equilibrating pH is the elution of 11 heme-components by Atassi (1964) from the chromatography of sperm whale myoglobin on CM-Cellulose columns. However, Atassi worked with the cyanmetmyoglobin derivative and his first three heme-components from the column were in the met form. Consequently, it is more difficult to evaluate his results. Also, Atassi is the only investigator to have reported better resolution on CM-Cellulose columns than on electrophoresis. He separated 11 heme-fractions on CM-Cellulose but only 4 components on electrophoresis.

However, none of these investigators mentions a FMF from CM-Cellulose columns. No fast-moving fraction is observed on electrophoresis of purified myoglobin. The FMF seems to be uniquely tied in with CM-Cellulose chromatography. On electrophoresis, the FMF, unlike purified myoglobin and other CM-Cellulose fractions, seems to contain components that streak at high pH values, and which seem to have an isoelectric point below 6.0. On repeated rechromatography on CM-Cellulose more than 90% of the FMF is converted each time into Mb I and II. This conversion to components of a more positive charge is not observed on electrophoresis of the FMF at pH 6.0. Furthermore, the 409/280 nm ratios of the rechromatographed FMF are lower than for the original sample. It was shown that the low ratios are not due to a dilution effect but might be due to contamination with light-scattering material picked up on the column. Finally, it has been shown that, under certain conditions, this FMF re-equilibrates on the column and is not eluted as a discrete fraction.

Similar conversions of one component to another on CM-Cellulose columns have been reported by Åkeson and Theorell (1960) for horse myoglobin, Atassi (1964) for sperm whale myoglobin, and Atassi and Saplin (1966) for finback whale myoglobin. Heterogeneity or interconversion of fractions from DEAE-Cellulose columns has been reported by all investigators using this column material. However, Edmundson and Hirs (1962a) did report chromatographic homogeneity of sperm whale myoglobin fractions from IRC-50 columns. No explanation for this difference is immediately available.

Atassi (1964) showed that polymerization of the protein is not involved in the conversion of one fraction to another. Evidence for a

pH-dependent polymerization of myoglobin has been presented in the literature. However, this was always shown to occur at pH values below 5; i.e. at pH values below those used in chromatography and electrophoresis; see Rumen and Appella (1962), and Strausser and Bucsi (1965). Hardman et al. (1966) were the only investigators to suggest aggregation of metmyoglobin at pH 6.6 to 7.5. They found evidence for this in chromatograms from G-50 and G-75 Sephadex columns. Their evidence shows 5% aggregation in myoglobin exposed to ammonium sulfate during purification, but only 1% aggregation in myoglobin isolated by the zinc-ethanol procedure. However, they did confirm the microheterogeneity of sperm whale myoglobin by chromatography and electrophoresis, and concur in the findings of numerous studies that indicate heterogeneity in the absence of aggregation.

Atassi (1964) showed "interconversion" of sperm whale myoglobin fractions on electrophoresis. Electrophoretic heterogeneity of CM-Cellulose fractions has also been shown by Atassi and Saplin (1966) for finback whale myoglobin, Åkeson and Theorell (1960) for horse myoglobin, Quinn et al. (1964) for bovine myoglobin fractions.

All investigators who used DEAE-Cellulose reported these fractions heterogeneous on electrophoresis: Perkoff et al. (1962) for human myoglobin; Quinn et al. (1964) for bovine myoglobin; Hardman et al. (1966) for sperm whale myoglobin; Åkeson and Theorell (1960) for horse myoglobin. These results are in substantial agreement with the findings in this study where it has been shown that all CM-Cellulose fractions are electrophoretically heterogeneous and that electrophoretic components can be further separated on electrophoresis in a second dimension.

It has been shown that e-1 is converted to e-1, e-2 and e-3 on

re-electrophoresis. It has also been shown that, at pH 6.0, the predominant electrophoretic components are: e-1 in purified myoglobin; e-1 in Mb I; e-2 in Mb II; e-3 and e-4 in the FMF, though small amounts of the other components are also present in each fraction. At pH values above 8.0 there is a change in the proportions of the electrophoretic components: purified myoglobin contains graded amounts of e-1 to e-4 with e-1 predominating; Mb I still contains mostly e-1 but has more e-2 and e-3 than at pH 6.0; Mb II has more e-1, e-3 and e-4 than at pH 6.0, and the FMF has more e-1 and e-2 than at pH 6.0. Furthermore, re-electrophoresis of e-1 at the same pH results in its conversion to e-1, e-2, e-3 and possibly e-4.

Chromatographic and electrophoretic results presented here indicate that myoglobin components undergo a pH dependent change in proportions.

Several reasons for the microheterogeneity of myoglobin have been proposed in the literature. Edmundson and Hirs (1962a) found no difference in amino acid content of the fractions from sperm whale myoglobin. However, they suggested that the differences may lie in variations of total amide content for the glutamic and aspartic residues. Hardman et al. (1966) concurred with the suggestion of a difference in primary structure. However, no concrete evidence of an amide difference was presented. In fact, Åkeson and Theorell (1960) ruled out a difference in total amide nitrogen for horse myoglobin fractions. They also found no difference in amino acid content. Rumen (1960) found no differences in amino acid content for seal myoglobin fractions. Gillespie et al. (1966) suggested as a possible explanation of microheterogeneity the binding of a phosphate ion to the distal E-7 histidine residue where a sulfate is bound in the

crystal.

The interconversion of fractions reported in this study is not compatible with a primary structure difference. However, it does suggest the possibility of differences in tertiary structure of the components. Such a difference in tertiary structure of the various myoglobin components is apt to be small since only small differences in spectra have been reported for the various fractions.

There is some evidence to support the theory of a difference in the tertiary structure of the various fractions. Rumen (1959) found a difference in isoelectric points of seal Mb I and II (Mb III, IV and V were not studied due to lack of protein). Vesterberg (1967) isolated horse myoglobin by the method of Åkeson and Theorell (1960). He showed that the three major purified fractions had pI values of 7.76, 7.32 and 7.26. He also showed that the first heterogeneous fraction eluted from a CM-Cellulose column contained three components with pI values of 6.89, 6.85 and 6.80. Allowing for species differences, the FMF in this study has been shown to differ from the other fractions in that it contains electrophoretic components with an isoelectric point below 6.0. The nature of these components in bovine myoglobin has not been determined.

Further evidence for a difference in configuration of Mb fractions has been presented by Breslow and Rumen (1967). Their titration studies showed that fraction V is missing 2 histidine residues normally exposed to solvent in fractions I and II. This evidence indicates a difference in tertiary structure.

Assumed differences in tertiary structure are compatible with results presented in this study. Åkeson and Theorell (1960) stated that a

difference in tertiary structure would explain their electrophoretic results. However, as they pointed out, a difference in tertiary structure of the protein does not explain the additional peptides found in their "fingerprints" of Mb I and Mb II_2. They suggest that a different distribution of the amide groups within the molecule could cause large enough shifts of dissociation constants of appropriate groups to account for differences in electrophoretic mobilities, and would also give rise to differences in peptide patterns. However, they suggest that a difference of constituents other than amino acids must be kept in mind.

A difference in the distribution of amide groups within the molecule, thus the existence of two similar species of peptides that differ only in charge would result in different rates of migration of the peptides, and their separation. However, if the same reasoning is applied to two co-existing species of myoglobin molecules in solution, it is difficult to see how either species, once separated, as for instance e-1, could convert into the other; or if they were in equilibrium one with the other, how they could be separated to begin with.

Åkeson and Theorell (1960) ruled out a difference in tertiary structure alone for two reasons: the fact that it does not explain the two additional peptides, and because the myoglobin components could not be separated on the ultracentrifuge. However, a configurational difference has to be rather large in order to obtain an ultracentrifugal separation (personal communication, Dr. Darrel E. Goll).

These pH-dependent changes in the proportions of myoglobin components are not compatible with a primary structure difference. However, they are indicative of differences in tertiary structure. Furthermore, the possi-

bility of the binding of a phosphate ion as suggested by Gillespie et al. (1966) has not been eliminated.

In conclusion, even though there seems to be no good biological reason for the existence of more than one myoglobin, the evidence for the microheterogeneity of this protein from different species, purified in different ways, is overwhelming. However, because of the numerous treatments to which the protein is subjected in the process of isolation and purification, one cannot entirely rule out the possibility of an artifact.

SUMMARY AND CONCLUSIONS

This study has shown that the microheterogeneity of bovine myoglobin is not caused by a protein-protein interaction of myoglobin with a non-heme contaminating protein; is not eliminated by 2-mercaptoethanol; is not due to the binding of metal ions; is not due to the acid-alkaline forms of the protein.

Evidence obtained in this study indicates that the number of bovine myoglobin fractions obtained from CM-Cellulose columns can be varied from 2 to 5 depending on the experimental conditions used. It has been shown that, under certain conditions, a fast-moving myoglobin fraction is eluted from CM-Cellulose columns. The size of this fast-moving fraction can be reduced at low pH values, and increased at high pH values.

Moreover, this fast-moving fraction, under certain conditions, re-equilibrates with the column material instead of being eluted as a discrete fraction. A rise in pH of the effluent is observed at the start of eluting a column. This rise in pH may contribute in part to the formation of the fast-moving fraction, and may be caused by the displacement of more basic potassium ions by less basically charged protein groups on the exchanger. Repeatedly, on rechromatography at pH 6.0, over 90% of the fast-moving fraction is converted to the components with a more positive charge. This conversion does not occur on electrophoresis of the fast-moving fraction at pH 6.0. Electrophoresis of purified myoglobin results in the separation of at least four, maybe five or six electrophoretic components, but not of a fast-moving component as observed on CM-Cellulose columns. The fast-moving fraction seems to be uniquely tied in with CM-Cellulose

chromatography. None of the CM-Cellulose fractions were found homogeneous on electrophoresis.

A pH-dependent shift in the proportions of myoglobin components was shown on electrophoresis. At pH 8.0 or above a larger proportion of the more negatively charged components are formed than at pH 6.0. At pH 6.0 or below, the more positively charged components predominate. On re-electrophoresis of the major electrophoretic component at the same pH, some of the fraction is converted into the minor components.

It was concluded that the interconversion of fractions is not compatible with a primary structure difference. However, the pH-dependent shift in the proportions of myoglobin fractions could be due to a difference in tertiary structure of the components. However, the possibility of the binding of an ion by some of the molecules has not been eliminated by the results presented in this study.

LITERATURE CITED

Åkeson, Åke. 1962. The C-terminal amino acids of three variants of horse myoglobin. Acta Chemica Scandinavica 16: 526-528.

Åkeson, Åke, Ehrenstein, G. V., Hevesy, G. and Theorell, Hugo. 1960. Life span of myoglobin. Archives of Biochemistry and Biophysics 91: 310-318.

Åkeson, Åke and Theorell, Hugo. 1960. On the microheterogeneity of horse myoglobin. Archives of Biochemistry and Biophysics 91: 319-325.

Aoki, K. and Foster, J. F. 1956. Electrophoretic demonstration of the isomerization of bovine plasma albumin at low pH. American Chemical Society Journal 78: 3538-3539.

Atassi, M. Z. 1964. Properties of components of myoglobin of the sperm whale. Nature 202: 496-498.

Atassi, M. Z. and Saplin, Barbara J. 1966. Studies on myoglobin from the finback whale (Balaenoptera physalus). Biochemical Journal 98: 82-93.

Banaszak, L. J., Andrews, P. A., Burgner, J. W., Eylar, E. H. and Gurd, Frank R. N. 1963a. Carboxymethylation of sperm whale metmyoglobin. Journal of Biological Chemistry 238: 3307-3314.

Banaszak, L. J., Eylar, E. H. and Gurd, Frank R. N. 1963b. Comparison of sperm whale metmyoglobin with its guanidinated derivative. Journal of Biological Chemistry 238: 1989-1994.

Banaszak, L. J. and Gurd, Frank R. N. 1964. Carboxymethylation of sperm whale metmyoglobin: reactivity of the adjacent histidine residues. Journal of Biological Chemistry 239: 1836-1838.

Benoit, F. L., Theil, C. B. and Watten, R. H. 1963. Foetal myoglobin in the urine of an adult. Nature 199: 387.

Benoit, F. L., Theil, G. B. and Watten, R. H. 1964. Myoglobinuria and persistence of fetal myoglobin. Annals of Internal Medicine 61: 1133-1139.

Beychok, S. and Blout, E. R. 1961. Optical rotatory dispersion of sperm whale ferrimyoglobin and horse ferrihemoglobin. Journal of Molecular Biology 3: 769-777.

Beychok, S., De Lozé, C. and Blout, E. R. 1962. Helix contents of solutions of native and acid-denatured ferrihemoglobin and ferrimyoglobin. Journal of Molecular Biology 4: 421-429.

Beychok, S. and Steinhardt, J. 1960. Distinct reversibly denatured ferrihemoglobins produced at pH 3.5. American Chemical Society Journal 82: 2756-2762.

Bodo, G., Dintzis, H. M., Kendrew, J. C. and Wyckoff, H. W. 1959. The crystal structure of myoglobin. V. A low-resolution three-dimensional Fourier synthesis of sperm whale myoglobin crystals. Royal Society Proceedings Series A, 253: 70-102.

Breslow, Esther. 1962. Role of the heme in the titration of sperm whale myoglobin. Journal of Biological Chemistry 237: PC3308-PC3311.

Breslow, Esther. 1964a. Changes in side chain reactivity accompanying the binding of heme to sperm whale apomyoglobin. Journal of Biological Chemistry 239: 486-496.

Breslow, Esther. 1964b. Comparison of cupric ion-binding sites in myoglobin derivatives and serum albumin. Journal of Biological Chemistry 239: 3252-3259.

Breslow, Esther, Beychok, Sherman, Hardman, Karl D. and Gurd, F. R. N. 1965. Relative conformations of sperm whale metmyoglobin and apomyoglobin in solution. Journal of Biological Chemistry 240: 304-309.

Breslow, Esther and Gurd, F. R. N. 1962. Reactivity of sperm whale metmyoglobin towards hydrogen ions and p-nitrophenyl acetate. Journal of Biological Chemistry 237: 371-381.

Breslow, Esther and Gurd, Frank, R. N. 1963. Interaction of cupric and zinc ions with sperm whale metmyoglobin. Journal of Biological Chemistry 238: 1332-1342.

Breslow, Esther and Koehler, Robert. 1965. Combination of Protoporphyrin IX with sperm whale apomyoglobin. Journal of Biological Chemistry 240: PC2266-PC2268.

Breslow, Esther and Rumen, Nevenka M. 1967. Reactivity of seal myoglobins toward hydrogen ions. Archives of Biochemistry and Biophysics 121: 50-54.

Cann, John R. 1963. Spectroscopic investigation of the reaction of sperm whale myoglobin with zinc. National Academy of Sciences Proceedings 50: 368-372.

Cann, John R. 1964a. Kinetics of the reversible reaction of sperm whale metmyoglobin with zinc. Biochemistry 3: 714-722.

Cann, John R. 1964b. Effect of ligands and oxidation state upon the reaction of myoglobin and hemoglobin with zinc. Biochemistry 3: 903-908.

Cann, John R. 1965. Contribution of aromatic residue interactions to the stability of myoglobin. Biochemistry 4: 2368-3272.

Clayton, James W. and Bushuk, Walter. 1966. Ion exchange chromatography of proteins; artifact separations resulting from inadequate pH control. Journal of Chromatography 21: 67-74.

Colvin, J. R., Smith, David B. and Cook, W. H. 1954. The microheterogeneity of proteins. Chemical Reviews 54: 687-711.

Criddle, R. S., Bock, R. M., Green, D. E. and Tisdale, H. D. 1961. Specific interaction of mitochondrial structural protein (S.P.) with cytochromes and lipid. Biochemical and Biophysical Research Communications 5: 75-80.

Dixon, Malcolm and Webb, Edwin C. Enzymes. Second ed. New York, N.Y., Academic Press, Inc. 1964.

DuFresne, Ann. 1964. Bovine myoglobin: differences between chromatograms of preparations from individual animals. Life Sciences 3: 1201-1206.

Edmundson, A. B. 1965. Amino acid sequence of sperm whale myoglobin. Nature 205: 883-887.

Edmundson, A. B. and Hirs, C. H. W. 1961. The amino acid sequence of sperm whale myoglobin. Nature 190: 663-672.

Edmundson, A. B. and Hirs, C. H. W. 1962a. On the structure of sperm whale myoglobin. I. The amino acid composition and terminal groups of the chromatographically purified protein. Journal of Molecular Biology 5: 663-682.

Edmundson, A. B. and Hirs, C. H. W. 1962b. On the structure of sperm whale myoglobin. II. The tryptic hydrolysis of the denatured protein. Journal of Molecular Biology 5: 683-705.

Edmundson, A. B. and Hirs, C. H. W. 1962c. On the structure of sperm whale myoglobin. III. Amino acid sequences of the smaller tryptic peptides containing aromatic residues. Journal of Molecular Biology 5: 706-708.

Feeney, Robert E. The evolution of proteins. In Schultz, H. W. and Anglemier, A. F., eds. Symposium on foods: proteins and their reactions. Pp. 345-359. Westport, Connecticut, The Avi Publishing Co., Inc. 1964.

George, P. and Hanania, G. 1952. The ionization of acidic metmyoglobin. Biochemical Journal 52: 517-523.

Gillespie, J. M., Hapner, K. D. Hartzell, C. R. and Gurd, F. R. N. 1966. Interaction of sperm whale metmyoglobin with phosphate. Journal of

Molecular Biology 21: 399-402.

Hardman, Karl D., Eylar, Edwin H., Ray, D. K., Banaszak, Leonard J. and Gurd, Frank R. N. 1966. Isolation of sperm whale myoglobin by low temperature fractionation with ethanol and metallic ions. Journal of Biological Chemistry 241: 432-442.

Harfenist, Elizabeth J. 1953. The amino acid compositions of insulins isolated from beef, pork and sheep glands. American Chemical Society Journal 75: 5528-5533.

Harfenist, Elizabeth J. and Craig, Lyman C. 1952a. Countercurrent distribution studies with insulin. American Chemical Society Journal 74: 3083-3087.

Harfenist, Elizabeth J. and Craig, Lyman C. 1952b. The molecular weight of insulin. American Chemical Society Journal 74: 3087-3089.

Harfenist, Elizabeth J. and Craig, Lyman C. 1952c. Differences in the quantitative amino acid composition of insulins isolated from beef, pork and sheep glands. American Chemical Society Journal 74: 4216-4217.

Harrison, S. C. and Blout, E. R. 1965. Reversible conformational changes of myoglobin and apomyoglobin. Journal of Biological Chemistry 240: 299-303.

Haut, A., Tudhope, G. R., Cartwright, G. E. and Wintrobe, M. M. 1962. The nonhemoglobin erythrocytic proteins, studied by electrophoresis on starch gel. Journal of Clinical Investigation 41: 579-587.

Ingram, V. M. 1956. A specific chemical difference between the globins of normal human and sickle-cell anaemia haemoglobin. Nature 178: 792-794.

Ingram, V. M. 1958. Abnormal human haemoglobins. I. The comparison of normal human and sickle-cell haemoglobins by "Fingerprinting". Biochimica et Biophysica Acta 28: 539-545.

Jonxis, J. H. P. and Wadman, S. K. 1952. A fetal form of myoglobin. Nature 169: 884-886.

Kendrew, J. C. 1963. Myoglobin and the structure of proteins. Science 139: 1259-1266.

Kendrew, J. C., Bodo, G., Dintzis, H. M., Parrish, R. G., Wyckoff, H. W. and Phillips, D. C. 1958. A three-dimensional model of the myoglobin molecule obtained by x-ray analysis. Nature 181: 662-666.

Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Phillips, D. C. and Shore, V. C. 1960. Structure of myoglobin:

a three-dimensional Fourier synthesis at 2 Å resolution. Nature 185: 422-427.

Kendrew, J. C. and Parrish, R. G. 1956. The crystal structure of myoglobin. III. Sperm-whale myoglobin. Royal Society Proceedings (London), Series A, 238: 305-324.

Kendrew, J. C., Parrish, R. G., Marrack, J. R. and Orlans, E. S. 1954. The species specificity of myoglobin. Nature 174: 946-949.

Kendrew, J. C., Watson, H. C., Strandberg, B. E., Dickerson, R. E., Phillips, D. C. and Shore, V. C. 1961. The amino acid sequence of sperm whale myoglobin. (II). A partial determination by x-ray methods, and its correlation with chemical data. Nature 190: 666-670.

Koltun, W. L., Clark, R. E., Dexter, R. N., Katsoyannis, P. G. and Gurd, F. R. N. 1959. Coordination complexes and catalytic properties of proteins and related substances. II. The reactivity of carbobenzoxy-L-prolyl-L-histidylglycinamide. American Chemical Society Journal 81: 295-301.

Koltun, W. L., Dexter, R. N., Clark, R. E. and Gurd, F. R. N. 1958. Coordination complexes and catalytic properties of proteins and related substances. I. Effect of cupric ions on the hydrolysis of p-nitrophenyl acetate by imidazole. American Chemical Society Journal 80: 4188-4194.

Koltun, W. L., Ng, Lionel and Gurd, F. R. N. 1963. Reactivity of peptide imidazole, amino, and phenolic groups towards p-nitrophenyl acetate. Journal of Biological Chemistry 238: 1367-1372.

Kossman, R. J., Fainer, D. C. and Boyer, S. H. 1964. A study of myoglobin in disease with comments concerning the myoglobin minor components. Cold Spring Harbor Symposium on Quantitative Biology 29: 375-385.

Lewis, U. J. 1954. Acid cleavage of heme proteins. Journal of Biological Chemistry 206: 109-120.

Lewis, U. J. and Schweigert, B. S. 1955. Biochemistry of myoglobin. III. Homogeneity studies with crystalline beef myoglobin. Journal of Biological Chemistry 214: 647-655.

Miyoshi, K., Saijo, K., Kuryu, Y. and Oshima, Y. 1963. Abnormal myoglobin ultraviolet spectrum in Duchenne type progressive muscular dystrophy. Science 142: 490-491.

Nobbs, C. L., Watson, H. C. and Kendrew, C. B. E. 1966. Structure of deoxymyoglobin: a crystallographic study. Nature 209: 339-341.

Okubo, Hideo. 1963. Purification of myoglobin. Kyushu Journal of

Medical Science 14: 431-445.

Paleus, Sven and Neilands, J. B. 1950. Preparation of cytochrome c with the aid of ion exchange resin. Acta Chemica Scandinavica 4: 1024-1030.

Perkoff, Gerald T. 1964. Studies of human myoglobin in several diseases of muscle. New England Journal of Medicine 270: 263-269.

Perkoff, Gerald T. 1965. Myoglobin. American Journal of Medicine 39: 527-532.

Perkoff, Gerald T. 1966. Evidence for a specific human fetal muscle heme protein. Journal of Laboratory and Clinical Medicine 67: 585-600.

Perkoff, Gerald T., Hill, Robert L., Brown, Douglas M. and Tyler, Frank H. 1962. The characterization of adult human myoglobin. Journal of Biological Chemistry 237: 2820-2827.

Perkoff, Gerald T. and Tyler, Frank H. 1958. Estimation and physical properties of myoglobin in various species. Metabolism 7: 751-759.

Perlmann, Gertrude E. 1949. The electrophoretic properties of plakalbumin. American Chemical Society Journal 71: 1146-1149.

Perlmann, Gertrude E. 1952. Enzymatic dephosphorylation of ovalbumin and plakalbumin. Journal of General Physiology 35: 711-726.

Perlmann, Gertrude E. 1953. Electrophoretic studies of enzymatically modified ovalbumin and casein. Faraday Society Discussions 13: 67-77.

Porter, R. R. and Sanger, F. 1948. The free amino groups of haemoglobins. Biochemical Journal 42: 287-294.

Poulik, M. D. 1957. Starch gel electrophoresis in a discontinuous system of buffers. Nature 180: 1477-1479.

Quinn, J. R. and Pearson, A. M. 1964. Characterization studies of three myoglobin fractions from bovine muscle. Journal of Food Science 29: 429-434.

Quinn, J. R., Pearson, A. M. and Brunner, J. R. 1964. Detection and isolation of multiple myoglobins from beef muscle. Journal of Food Science 29: 422-428.

Raymond, Samuel and Aurell, Barbo. 1962. Two-dimensional gel electrophoresis. Science 138: 152-153.

Raymond, Samuel and Nakamichi, Masumi. 1962. Electrophoresis in synthetic gels. I. Relation of gel structure to resolution. Analytical

Biochemistry 3: 23-30.

Raymond, Samuel and Wang, Yi-Ju. 1960. Preparation and properties of acrylamide gel for use in electrophoresis. Analytical Biochemistry 1: 391-396.

Rossi-Fanelli, S., Antonini, E., de Marco, C. and Bernedetti, S. Studies on foetal myoglobin. In Wolstenholme, G. E. W. and O'Connor, C. M., eds. Biochemistry of human genetics. P. 144. Boston, Mass., Little Brown and Co. 1959.

Rumen, Nevenka M. 1959. Isolation of five myoglobins from seal (Phoca vitulina). Acta Chemica Scandinavica 13: 1542-1546.

Rumen, Nevenka M. 1960. Amino acid composition of seal myoglobin I. Acta Chemica Scandinavica 14: 1217-1218.

Rumen, Nevenka M. and Appella, Ettore. 1962. Molecular association behavior of apomyoglobin I from the seal (Phoca vitulina). Archives of Biochemistry and Biophysics 97: 128-133.

Samejima, Tatsuya and Yang, Jen Tsi. 1964. Optical rotatory dispersion of sperm-whale myoglobin and its derivatives. Journal of Molecular Biology 8: 863-871.

Schmid, K. 1949. Electrophoretic properties and analysis of whale myoglobin. Nature 163: 481-482.

Schneiderman, L. J. 1962. Myoglobin in the human foetus. Nature 194: 191-192.

Singer, K., Angelopoulous, B. and Ramot, B. 1955. Studies on human myoglobin. II. Fetal myoglobin: its identification and its replacement by adult myoglobin during infancy. Blood 10: 987-998.

Snyder, Harry E. and Ayres, John C. 1961. The autoxidation of crystallized beef myoglobin. Journal of Food Science 26: 469-474.

Sogami, M. and Foster, J. F. 1963. Microheterogeneity as the explanation for resolution of N and F forms of plasma albumin in electrophoresis and other experiments. Journal of Biological Chemistry 238: PC2245-PC2247.

Stockwell, Anne. 1961. Comparative studies on tryptic digests of myoglobins. Journal of Molecular Biology 3: 362-366.

Strausser, H. R. and Bucsi, R. A. 1965. Immunochemical studies of human myoglobin heterogeneity. American Zoologist 5: 639-640.

Stryer, L., Kendrew, J. C. and Watson, H. C. 1964. The mode of attachment of the azide ion to sperm whale metmyoglobin. Journal of Molecular

Biology 8: 96-104.

Synge, R. L. M. 1949. Naturally occurring peptides. Quarterly Reviews (London) 3: 245-262.

Theil, G. B. and Williams, J. K. 1967. Characteristics of adult and foetal myoglobin in the visible light spectrum. Nature 213: 75-76.

Theorell, A. Hugo T. 1932. Kristallinisches myoglobin. I. Kristallisieren und reinigung des myoglobin sowie verlaufige mitteilung über sein molekulargewicht. Biochemische Zeitschrift 252: 1-7.

Theorell, Hugo and Åkeson, Åke. 1955. Reversible splitting of a homogeneous horse myoglobin. Suomalaisen Tiedeakatemian Toimituksia (Annales Academiae Scientiarum Fennicae) Series A, II: Chemica, 60: 303-312.

Theorell, Hugo and Ehrenberg, A. 1951. Spectrophotometric, magnetic and titrimetric studies on the heme-linked groups in myoglobin. Acta Chemica Scandinavica 5: 823-848.

Timmer, R., Helm, H. J. Van der and Huisman, T. H. 1957. Amino acid composition of adult and foetal myoglobin of the cow. Nature 180: 239-240.

Urnes, P. J., Imahori, K. and Doty, P. 1961. The optical rotatory dispersion of right-handed alpha-helices in sperm whale myoglobin. National Academy of Sciences Proceedings 47: 1635-1641.

Vesterberg, Olof. 1967. Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. V. Separation of myoglobins and studies on their electro-chemical differences. Acta Chemica Scandinavica 21: 206-216.

Vesterberg, Olof and Svensson, Harry. 1966. Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. IV. Further studies on the resolving power in connection with separation of myoglobins. Acta Chemica Scandinavica 20: 820-834.

White, Abraham, Handler, Phillip and Smith, Emil L. Principles of biochemistry. Third ed. New York, N.Y., McGraw-Hill Book Co., Inc. 1964.

Williams, Edward J. 1966. Exposure of tryptophyl residues of metmyoglobin in solution. Archives of Biochemistry and Biophysics 115: 21-26.

Whorton, C. M., Hudgins, P. C. and Connors, J. J. 1961. Abnormal spectrophotometric absorption spectrums of myoglobin in two forms of progressive muscular dystrophy. New England Journal of Medicine 265: 1242-1245.

Whorton, C. M., Hudgins, P. C., Connors, J. J. and Nadas, S. 1963. The myoglobin molecule and its possible structural alterations in diseased states of skeletal and cardiac muscle. Southern Medical Journal 56: 583-587.

Wolfson, R., Yakulis, V., Coleman, R. D. and Heller, P. 1967. Studies on fetal myoglobin. Journal of Laboratory and Clinical Medicine 69: 728-736.

ACKNOWLEDGMENTS

The author wishes to acknowledge the many students and faculty members who, directly or indirectly, helped shape this study. The many helpful suggestions of Dr. Alfred Mirna, Dr. T. W. Kwon and Dr. W. Duane Brown were especially appreciated.

Particular thanks are due to Dr. Harry E. Snyder whose unwaning patience and guidance throughout this study will long be remembered. His efforts to instill an interest in research for the sake of research will, without a doubt, be fruitful. The word "THINK" will haunt me for years to come.

Special thanks are also due to Dr. Dorris A. Lillard for repairing the fraction collector when the need was great, and others failed. Without his many helpful suggestions and encouraging words this study may never have been completed.

It is difficult, if not impossible, to express adequate thanks to Dr. Allen A. Kraft who helped in many ways above and beyond the call of duty.

Finally, the author wishes to express her sincere thanks to Dr. John C. Ayres who so graciously gave her the opportunity to undertake this study, and to all the members of her committee for seeing her through this final agony.