The Characterization of Adult Human Myoglobin*

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It has been reported that crystalline human myoglobin can be resolved by electrophoresis on paper into three components (1, 2).

Two of these possess nearly identical absorption spectra between 500 and 600 nm and give the same reactions with oxygen. Although it was suggested that these components differ structurally (2), no evidence has been presented to support this view.

In the present study the heterogeneity of human metmyoglobin has been examined with the aid of ion exchange chromatography. Five chromatographically distinct fractions, two of which represent approximately 75% of the myoglobin in skeletal muscle, have been found. Chemical and physical analyses of these fractions indicate that the globin is identical in the two myoglobins that are present in major amount. The differences in chromatographic and electrophoretic behavior of these fractions result from differences in the state of the iron in the heme prosthetic group. At least one of the other myoglobin components, which represents only a minor amount of the myoglobin of normal muscle, appears to differ in the primary structure of the globin moiety. The availability of large amounts of chromatographically pure myoglobin has prompted further characterization of this protein.

**EXPERIMENTAL PROCEDURE**

**Materials and Methods**—The myoglobin that was used as the starting material for the chromatographic experiments was prepared from skeletal muscle by a modification of the methods of Drabkin (3) and Ginger, Wilson, and Schweigert (4) as described previously (5). The muscle was obtained at autopsy, within 4 to 14 hours of death, from patients who died of nonmuscular disorders and was stored at -5° until used. Crystalline myoglobin was prepared by the method of Lugnagh (6). Spectral analysis showed that the myoglobin in these preparations was in the ferri- or met-form.

Dilute solutions of myoglobin were concentrated by one of three means. Saturation of aqueous solutions with ammonium sulfate at pH 7 completely precipitates myoglobin, which then can be collected by gravity filtration on fluted filter paper, suspended in a small amount of water, and dialyzed until free of ammonium sulfate. Concentration also was obtained by centrifugation at 96,000 × g in the Spinco model L ultracentrifuge at 4° for 72 hours. Under these conditions, myoglobin sediments to the bottom of the centrifuge tube and can be removed from the tube with a needle and syringe. The most effective means for concentrating myoglobin from very dilute solutions (< 0.2%) was by adsorption on DEAE-cellulose. Myoglobin is adsorbed quantitatively from solutions (< 0.001 M in salt concentration, pH 8.9) at 6° on DEAE-cellulose that has been equilibrated with 0.001 M Tris buffer, pH 8.9. Adsorption is most effective when the cellulose is packed in columns with dimensions of approximately 1 × 10 cm. The myoglobin is eluted from the cellulose with 1 M Tris buffer, pH 7.85, or 1 M sodium chloride (unbuffered). Buffer salts or sodium chloride are removed by dialysis.

The concentration of myoglobin was measured spectrophotometrically. The extinction coefficient (ε in cm⁻¹ liter·mole⁻¹) of human myoglobin was determined for several preparations from simultaneous dry-weight determinations at 105° and spectrophotometric analysis at 280 nm. For a molecular weight of 17,500, the molar extinction coefficient is 3.4 × 10⁴ between pH 5 and 9.

Zone electrophoresis on starch gels was performed by the method of Smithies (7). Starch gels were stained with Amido Black B (7) and with benzidine hydrochloride (8). Two-dimensional paper electrophoresis-chromatography of tryptic peptides was performed as described earlier (9). Spectral analyses were made with a Carey Model 14 automatic recording spectrophotometer. Amino terminal end group analyses were performed by the phenyl isothiocyanate and the fluorodinitrobenzene techniques (10). Carboxyl-terminal end group analysis was made with carboxypeptidase A. Amino acid analyses were performed with the Spinco model MS automatic amino acid analyzer (11, 12). ¹⁸⁶ Iron was estimated by the method of Gubler et al. (13). Carboxypeptidase A (Worthington Biochemical Corporation, 3 X crystallized) was treated with diisopropyl fluorophosphate before use. Crystalline trypsin was a commercial preparation (Worthington). ¹⁸⁶

Chromatography of Myoglobin—Myoglobin was chromatographed at 6° on DEAE-cellulose that had been equilibrated with 0.005 M Tris buffer, pH 7.85. Jacketed columns (0.9 × 12 cm) which were cooled by circulation of water at 6° were used for analysis of small (5 to 15 mg) amounts of myoglobin. Chromatography on a preparative scale (0.5 to 1.2 g) was achieved in the same manner with 5 × 15-cm jacketed columns. Typical results of a chromatographic separation of myoglobin performed on preparative columns are shown in Fig. 1. Four fractions (Fᵡ, Fᵢ, Fᵢ₉, Fᵢ₅) were eluted with the dilute Tris buffer, whereas

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Fraction varied among different preparations; however, F1, Fz, and Fb were developed with 1 chromato- 
graphic pattern as the noncrystalline myoglobin shown in Fig. 1. The small cyanmetmyoglobin, only one peak was observed in addition to another. This is seen in Fig. 2A (FI and Fz oxymyoglobins) and B (FI and Fz cyanmetmyoglobins). The small differences in the point of emergence probably reflect variations in the size of the columns used in the experiments. When un- fractionated preparations were chromatographed as oxymyoglobin and cyanmetmyoglobin, they could not be distinguished from one form. When FI and Fs were chromatographed as oxymyoglobin in each fraction can be converted into the other chromatographic form. Although it is evident that FI shows a considerable amount of PI, this result is analogous to one of the major chromatographic fractions (F1, F2, and F3). The heme protein that moves more rapidly than the major components in unfract-ionated preparations was found in F3 and presumably represents one of the minor components of this fraction (Fig. 3). Although it is evident that F1 shows a considerable amount of F2, and F2 shows significant amounts of F3, this result is analogous to the behavior of these fractions on rechromatography. Fraction B could not be distinguished from F3 by this method. It is noteworthy that F3 has a greater electrophoretic mobility than any of the other fractions. When unfract-ionated myoglobin was analyzed as described above except that the columns were pooling the myoglobin emerging in the peak tubes.

FIG. 1. Chromatography of human myoglobin on DEAE-cellulose. Myoglobin, 800 mg, dissolved in 50 ml of water was dialyzed for 18 hours at 5° against 50 volumes of 0.005 M Tris buffer, pH 7.85, and applied to a column (5 X 12 cm) of DEAE-cellulose which was previously equilibrated with the same buffer. The flow rate was 60 ml per hour; the temperature was maintained at 6° until 8.2 liters were collected. The column then was warmed to 22° and developed with 1 M Tris buffer, pH 7.85. Ten-milliliter fractions were collected and analyzed spectrophotometrically at 415 and 280 m. Further analysis of each component was made by electrophoresis on starch gels. One (F3) was eluted with 1 M Tris buffer, pH 7.8, at 25°C. Fractions F1, F2, and F3 contained the majority of the heme protein. Fraction A, which emerged from the column at the hold-up volume, contained only none-heme protein. The yield of each fraction varied among different preparations; however, F1, F2, and F3 generally accounted for 55 to 65%, 15 to 20%, and 15 to 30%, respectively, of the total myoglobin applied to the column. It is noteworthy that the fractions had visibly different colors: F1 was brown, F3 was dark red, F5 was reddish-brown, and F6 was greenish-brown. Crystalline myoglobin gave the same chromatographic pattern as the noncrystalline myoglobin shown in Fig. 1.

When FI and Fz were rechromatographed separately under the same conditions, two components were observed. Eighty per cent of F1, emerged from the column in the volume expected from its behavior as shown in Fig. 1, whereas the remainder behaved like Fz. Fraction 2 on the other hand revealed approximately 25% of Fi on rechromatography. Because the rechromatographed samples could not be contaminated with one another to this extent, these results suggest that the myoglobin in each fraction can be converted into the other chromatographic form. When Fi and Fz were chromatographed as oxymyoglobin or cyanmetmyoglobin, they could not be distinguished from one another. This is seen in Fig. 2A (FI and Fz oxymyoglobins) and in Fig. 2B (FI and Fz cyanmetmyoglobins). The small differences in the point of emergence probably reflect variations in the size of the columns used in the experiments. When unfract-ionated preparations were chromatographed as oxy- or cyanmetmyoglobin, only one peak was observed in addition to F3.

Fraction 3 could not be purified further under the conditions described in Fig. 1. The most successful means for separating the myoglobin in this fraction was by chromatography as shown in Fig. 3. A gradient of sodium chloride formed with the cone- 

sphere arrangement (1850 ml, total volume) described by Sober and Peterson (14) was used in conjunction with a DEAE-cellulose column and 0.005 M Tris, pH 7.85, as the starting buffer. Six distinct fractions were obtained, only three of which were present in significant amounts (F1A, F1B, and F3B). Because each component except F3B had a smaller (415/280 mp) absorption ratio than F1 and F2, it is likely that significant amounts of non-heme proteins contaminated all fractions but F3B. Attempts to purify the other heme proteins in F1 by varying the chromatographic conditions were unsuccessful.

Electrophoresis of Myoglobin on Starch Gels—Zone electrophoresis on starch gels proved to be a useful means of characterizing unfract-ionated myoglobin. Fig. 4 shows the electrophoretic behavior of unfract-ionated myoglobin and of the chromatograph- 
ically purified fractions. Three heme proteins could be distinguished. Occasionally a fourth, which migrated more rapidly than the others, was observed. Each of the three major heme proteins in unfract-ionated myoglobin was similar to one of the major chromatographic fractions (F1, F2, and F3). When F1 and Fs were chromatographed as oxymyoglobin, the fractions had visibly different colors: F1 was brown, F3 was dark red, and F5 was reddish-brown. The heme protein that moves more rapidly than the major components in unfract-ionated preparations was found in F3 and presumably represents one of the minor components of this fraction (Fig. 3). Although it is evident that F1 shows a considerable amount of F2, and F2 shows significant amounts of F3, this result is analogous to the behavior of these fractions on rechromatography. Fraction B could not be distinguished from F3 by this method. It is noteworthy that F3 has a greater electrophoretic mobility than any of the other fractions. When unfract-ionated myoglobin was analyzed as described above except that the columns were pooling the myoglobin emerging in the peak tubes.

FIG. 2. Rechromatography of purified fractions of myoglobin. Samples of F1 and F5, which were obtained as shown in Fig. 1, were applied to 0.9 X 12 cm columns of DEAE-cellulose and developed at 6° with Tris buffer at a flow rate of 20 ml per hour. Fractions, 3 ml, were collected, and the myoglobin was estimated spectrophotometrically at 415 mp. Each fraction was chromatographed on separate columns. A, Chromatography of F1 and F5 as oxymyoglobin. Eight milligrams of each fraction, dissolved in 4 ml of Tris buffer, were treated with 0.5 mg of sodium dithionate and oxygenated for 20 minutes with purged oxygen. Each solution was then dialyzed against buffer which was previously saturated with oxygen. B, Chromatography of F1 and F5 as cyanmet- 
myoglobin. Ten to twelve milligrams of each fraction, dissolved in 4 ml of Tris buffer, were converted to the cyanide derivative by mixing with 0.5 ml of 0.0012 M potassium cyanide. Each fraction was analyzed as described above except that the columns were developed with the Tris buffer containing 0.001 M potassium cyanide.
FIG. 3. Rechromatography of myoglobin \( F_1 \). \( F_2 \), 60 mg, which was obtained as shown in Fig. 1, was converted to cyanometmyoglobin as described in Fig. 2B. The myoglobin was then applied to a 1 \( \times \) 10 cm column of DEAE-cellulose which had been equilibrated with 0.005 M Tris buffer, pH 7.8, containing 0.001 M potassium cyanide at 6°C. After the sample was absorbed at the top of the column, the column was developed at 6°C by gradient elution as described in the text. Fractions of 5 ml were collected at a flow rate of 20 ml per hour.

Bin and each of the chromatographic components were converted to cyanometmyoglobin and then analyzed electrophoretically, only 2 heme proteins ("\( F_1 \), \( F_2 \)" and \( F_3 \)) were demonstrable. This observation is consistent with the finding that \( F_1 \) and \( F_2 \) in the cyanmet-form emerge from columns of DEAE-cellulose in the same volume.

Comparison of Tryptic Peptides from Chromatographically Purified Myoglobins—In order to determine whether the chromatographically pure myoglobins differed from one another in primary structure, tryptic digests of each myoglobin were analyzed on paper by a two-dimensional electrophoretic-chromatographic technique (9). Myoglobin, 25 mg, dissolved in 2 to 3 ml of water, were treated at -10°C with 25 volumes of an acetone-HCl mixture according to the methods of Rossi-Fanelli and Antonini (15). The resulting globin was dissolved in 2.5 ml of water, and the pH of the solution was adjusted to 8.0. Trypsin, 0.5 mg, dissolved in 0.1 ml of 0.001 N HCl, was added to start
FIG. 5. Two-dimensional peptide patterns of the tryptic peptides from human myoglobins F₁ and F₂. An aliquot of the tryptic digest which contained the peptides from 1 to 2 mg of myoglobin was resolved electrophoretically on Whatman 3MM paper for 3 hours at 10 volts per cm in pyridine acetate buffer, pH 6.5. The peptides then were resolved chromatographically in the second dimension for 20 hours with butanol-acetic acid-water (200:30:75). Peptides were located with a ninhydrin spray reagent.

the digestion and the mixture was incubated at 40°. Throughout the digestion, the pH was maintained between pH 7.8 and 8.2 by the addition of 0.1 N NaOH. The precipitate of globin disappeared during the first 15 to 30 minutes and hydrolysis was complete after 90 minutes. No differences were observed in the rates of hydrolysis of the different myoglobin fractions. Analysis of aliquots of the tryptic digests obtained in this manner gave peptide patterns like those shown in Fig. 5. Fractions 1, 2, and B were indistinguishable by this method of analysis. At least 24 distinct peptides were found, a number close to that expected from the lysine and arginine content of human myoglobin (Table I). Fractions 1, 2, and B also gave identical patterns when the papers were sprayed with reagents which specifically detect histidine (16), tryptophan (17), arginine (18), tyrosine (19) and sulfur-containing peptides (20). Fraction 3D (Fig. 3), which was the only myoglobin from Fraction 3 that was not contaminated with non-heme protein, gave patterns that differed from those in Fig. 5. Several spots in addition to those peptides found in Fractions 1, 2, and B were observed. This result, together with the difference in electrophoretic mobility noted when F₃ and F₁ were compared by zone electrophoresis, suggests that the primary structure of the myoglobin in F₃₅ may differ from that of F₁, F₂, and F₄. However, since F₃₅ may contain small amounts of non-heme protein, no definite conclusions about its primary structure can be drawn at this time. Additional experiments will be necessary to clarify the nature of this myoglobin.

PHYSICAL CHARACTERIZATION

Spectral Analysis—The ultraviolet and visible spectra of each of the myoglobin components were determined. The wave lengths of maximal absorption for four of the chromatographically purified myoglobins are listed in Table II. F₁ has absorption characteristics of acid metmyoglobin, whereas F₂ and F₃ have those of alkaline metmyoglobin (21). A portion of the spectra obtained with these types of metmyoglobin is shown in Fig. 6. The spectra of F₁ and F₂ were identical when they were converted to either the reduced, oxy-, cyanmet-, or carboxy-forms.

Sedimentation and Diffusion Studies—These analyses were made in 0.1 M Veronal buffer, pH 8.5, with the Spinco model E ultracentrifuge; each run was made at 59,780 r.p.m., 20°. The photographic plates were read with a microcomparator. All other operations were the same as reported in earlier studies from this laboratory (22, 23).

F₁ and F₂ sedimented as single, homogenous peaks. Their rate of sedimentation was a function of the protein concentration. Although the red color of myoglobin at concentrations greater than 6 mg per ml prevented more extensive studies, the Sₑₑₑ value at infinite dilution was calculated from the data available to be 1.815S.

Diffusion studies were performed in the Spinco model H electrophoresis-diffusion apparatus with the aid of the Rayleigh interference optical system. Diffusion constants were calculated by the methods of Longsworth (24) and Schachman (25). F₁ was analyzed in 0.1 M Veronal buffer, pH 8.5, at a protein concentration of 1.1 mg per ml. The color of the myoglobin prevented measurements at higher concentrations. Photographs of the interference patterns were calculated between 20 and 56 hours after formation of the boundary. The patterns obtained were read on a microcomparator. The results were corrected for viscosity, density, and temperature in the usual...
TABLE I
Amino acid composition of human myoglobin

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amino acid per 100 g of protein</th>
<th>Amino acid residuce per 100 g of protein</th>
<th>% as per cent of total N</th>
<th>Calculated no. of residues</th>
<th>Assumed no. of residues</th>
<th>No. of residues (Ref. 33)</th>
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<td>Alanine</td>
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<td>5.22</td>
<td>11.2</td>
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<td>Arginine</td>
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<td>5.80</td>
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<td>6.98</td>
<td>14.6</td>
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<td>13</td>
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<td>13.31</td>
<td>9.3</td>
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<td>16.3</td>
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<tr>
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<td>5.54</td>
<td>3.16</td>
<td>6.6</td>
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<tr>
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<td>3.37</td>
<td>2.94</td>
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<td>Serine</td>
<td>4.65*</td>
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<tr>
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<td>2.94*</td>
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<td>Tyrosine</td>
<td>3.60†</td>
<td>3.28</td>
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<td>Valine</td>
<td>2.35</td>
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<td>Alanine</td>
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<td></td>
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<tr>
<td>Total</td>
<td>114.04</td>
<td>98.31</td>
<td>102.45</td>
<td>154</td>
<td>150</td>
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* These values obtained by extrapolation to zero time hydrolysis.
† These values obtained from analysis of complete enzymic hydrolysates.
‡ These values omitted from the total.

The amino acid composition of myoglobin, presented in Table I, was determined by the method of Spackman, Stein, and Moore (11, 12). The results show a high proportion of the essential amino acids, particularly the sulfur-containing amino acids, which are important for the stability and functionality of the protein. The amino acid content reported in Table I is consistent with the known structure of myoglobin and its role in oxygen binding and transport.

CHEMICAL CHARACTERIZATION

Amino Terminal End-group Analysis—Globin, prepared from F1 and F2, gave glycine as the only detectable end group. Hydroxylamine, 0.05 ml, in 5 ml of ethanol was added to 2 ml of 1% sodium bicarbonate containing 25 mg of globin. After the mixture had reacted for 2 hours at 25°, the insoluble dinitrophenylglobin was collected by centrifugation, washed three times successively with water, ethanol, and ether, and then air-dried for 5 days. Samples of the dinitrophenylglobin were hydrolyzed with the formic acid-acetic anhydride-perchloric acid mixture of Hanes, Hird, and Isherwood (26) for 2 hours at 100°. Chromatographic analysis of hydrolysates (10) revealed only dinitrophenylglycine in an amount corresponding to 0.73 moles per mole of dinitrophenylglobin (mol. wt. 17,500). Because this value was not corrected for the weight of the dinitrophenyl groups in the protein, it is evident that the yield of dinitrophenyl glycine is close to 1 mole per mole of globin.

Similar results were obtained when globin was analyzed on paper strips (1 × 10 cm) with the phenyl isothiocyanate method described by Shelton and Schroeder (27). Only glycine phenyl isothiohydantoin was detected on Solvents A, D, and F of Sjögquist (28) and Edman and Sjögquist (29). Although no quantitative estimation was made of the amount of this derivative, the absence of other detectable phenyl thiohydantoins precludes the possibility of other amino end groups.

Carboxyl Terminal End-group Analysis—Globin is not sufficiently soluble at pH 8 to allow uniform digestion with carboxypeptidase A. Digestion was achieved, however, under conditions similar to those used by Guidotti for the a- and b-chains of human hemoglobin (30). Globin, 25 mg, dissolved in 2.5 ml of water with 0.125 ml of 0.2 M sodium lauryl sulfate and the pH of the mixture was adjusted to 8. After addition of 0.6 mg of carboxypeptidase A (0.05 ml) the mixture was incubated at 40°. Aliquots, 0.7 ml, were removed at 15, 30, and 55 minutes after addition of the enzyme, and acidified to stop hydrolysis.

The amino acid content of each aliquot was measured by the methods of Spackman, Stein, and Moore (11, 12). Fig. 7 shows the rate of release of the amino acids. Although it is impossible to distinguish between glutamic, asparagine, and glycine on the columns used for analysis, only glutamine was seen on paper chromatograms at 15, 30, and 55 minutes. It is evident that the

![Fig. 6. The absorption spectra of myoglobin Fractions 1 and 2.](http://www.jbc.org/content/237/9/2824.short/fig6)

TABLE II
Absorption maxima of human myoglobins

<table>
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<th>Myoglobin</th>
<th>Absorption maxima (wave length, m)</th>
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<tr>
<td>Fraction 1</td>
<td>631, 500, 410, 275</td>
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<tr>
<td>Fraction 2</td>
<td>580, 543, 412, 279</td>
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<tr>
<td>Fraction 3</td>
<td>580, 540, 414, 278</td>
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<tr>
<td>Fraction 5</td>
<td>540, 418, 273</td>
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<tr>
<td>Fraction 1, 2 (ferromyoglobin)</td>
<td>551, 424, 275</td>
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<tr>
<td>Fraction 1, 2 (oxyhemoglobin)</td>
<td>580, 540, 417, 282</td>
</tr>
<tr>
<td>Fraction 1, 2 (carboxymyoglobin)</td>
<td>580, 542, 522, 274</td>
</tr>
</tbody>
</table>

The absorption spectra of myoglobin Fractions 1 and 2 are shown in Fig. 6. The spectra exhibit characteristic features of myoglobin, including the Soret band at 409 nm and the Q bands at 577 and 555 nm. These spectral characteristics are consistent with the known structure of myoglobin and its role in oxygen binding and transport.
The tryptic digest of globin possesses the sequence Glu-Leu-Gly-Gly. However, it is apparent that more than one residue of glycine is released, which suggests that this amino acid occurs in more than one position in the carboxyl terminal sequence. Indeed, unpublished studies,2 have shown that a peptide from a tryptic digest of globin possesses the sequence Glu-Leu-Gly-Gly. However, it is apparent that more than one residue of glycine is released, which suggests that this amino acid occurs in more than one position in the carboxyl terminal sequence.

Tryptic digest of globin: Glu-Leu-Gly-Gly.

The iron content was 0.318%. Moisture content was 7.3%. Samples were prepared for hydrolysis by the method of Moore, Spackman, and Stein (12). When destruction of amino acids occurred during acid hydrolysis, the yields at different times of hydrolysis were corrected by linear extrapolation to zero time (34). Globin obtained from the myoglobin of F1 also was hydrolyzed completely with enzymes as described by Hill and Schmidt (35). Analysis of this enzymic hydrolysate provided an estimate of the tryptophan, asparagine, and glutamine content.

The composition of human myoglobin estimated by these methods is given in Table II. The weight recovery of amino acid residues and the nitrogen recovery, which are not corrected for the heme content of myoglobin, are within the precision of the analytical methods employed. The number of residues was calculated from the minimal molecular weight that was obtained with each of the amino acids on the basis of a molecular weight of 17,450. The molecular weight computed from the assumed number of residues is 17,330. When corrected for the heme content of myoglobin, a molecular weight of 17,260 is obtained, in close agreement with the value 17,900 estimated from sedimentation-diffusion measurements, and the value 17,450 calculated from the iron content.

It is noteworthy that the composition obtained by these methods differs somewhat from that reported earlier by Rossi-Fanelli, Cavallini, and DeMarco (33). The improved methods of chromatographic analysis of amino acids that were used here were not available when the earlier analyses were made, nor was a chromatographically purified myoglobin preparation examined at that time. These factors may account for the small differences observed. One analysis of a hydrolysate of F1 (see above) gave essentially the same composition shown in Table I for F1. F3 was not analyzed since its composition would be altered by the non-heme proteins that are present in this material.

*Unpublished studies, R. L. Hill. With the aid of the paper strip phenyl isothiocyanate end-group method (26), the amino terminal sequence in human myoglobin was found to be Gly-Leu-Ser-(Asp- or Glu-).
In contrast to $F_1$, $F_2$, and $F_3$, $F_4$ appears to contain more than one myoglobin component as well as non-heme proteins. When $F_4$ is analyzed electrophoretically on starch gels (Fig. 4), it migrates at pH 8.6 faster than either $F_1$ or $F_2$. As isolated from DEAE-cellulose (Fig. 1), it has a spectrum similar to that of alkaline metmyoglobin. Although it readily reacts with cyanide, as well as with oxygen or carbon monoxide after reduction with dithionite, its electrophoretic mobility is unaffected. Rechromatography, with the use of a salt gradient with DEAE-cellulose (Fig. 3) at pH 7.8, partially resolves $F_4$ into six different components. Despite these differences in the properties of $F_8$, the presence of small quantities of contaminating protein in $F_3$, the only $F_4$ component with a 415/280 nm ratio similar to $F_1$ or $F_2$, prevent definite conclusions to be made about primary structural differences.

It is interesting to compare the chromatographic behavior of human myoglobin with the behavior of myoglobin from other species. Brown (37) reported recently the chromatographic analysis of whale, bovine, and tuna myoglobins on DEAE-cellulose under conditions similar to those developed here. At pH 8.2 to 8.6 in 0.005 to 0.05 M Tris buffer, the bovine and whale myoglobins chromatographed as single, apparently homogeneous proteins. Bluefin tuna myoglobin, however, shows three peaks, each of which is analogous both in point of emergence and in amount to one of the three major peaks in human myoglobin. Lack of detailed studies of the three components prevents more detailed comparison. Chromatographic characterization of the myoglobin from other species has been achieved with carboxylic acid ion-exchange resins or celluloses. Rumen (38) has shown that five distinct components can be resolved from whale myoglobin at pH 8.6, all of which are reported to possess the same spectral properties. From the limited data presented it is impossible to judge whether any of these components correspond to acid or alkaline metmyoglobins. Edmundson and Hirs (39), with Amberlite IRC-50 at pH 5.86, also found five components in whale myoglobin. Because only acid metmyoglobin would be present at this pH, the heterogeneity of whale myoglobin appears to result at least in part from primary structural differences (39). Similarly, Ackeson and Theorell (40) have shown that equine myoglobin can be resolved into at least three components at pH 0.9 on carboxymethyl cellulose. This heterogeneity also appears to result from primary structural differences.

Although the properties of human myoglobin that have been determined in this study are similar to those of myoglobins from other species, certain structural differences between human myoglobin and other myoglobins are evident. The amino terminal and carboxyl terminal sequences of four species are shown in Table III. Certain species variations occur although none of these would be predicted to alter markedly properties of the myoglobin. The tryptic peptide patterns show distinct differences from those of other myoglobins that have been analyzed. Stockell (41) has compared the peptide patterns of seven species (5 mammals, 1 bird, and 1 reptile) and found considerable similarity in certain unique peptides. The methods employed here for the preparation of peptide patterns differ from those used by Stockell and direct comparison is impossible. The patterns of whale3 and human myoglobin prepared by our methods are shown in Fig. 8. Several similarities are evident among the peptides. On the basis of specific color reactions which certain peptides give for histidine, tryptophan, arginine, or tyrosine, at least eight peptides would seem to correspond to one another in the two myoglobins. The similar peptides are given identical numbers in the figure. The other peptides cannot be related easily to one another although the fact that human myoglobin does not yield an insoluble residue after tryptic digestion accounts for the larger number of peptides seen on the human pattern. Whereas these results are not unexpected, it is surprising that the amino acid composition of whale (39) and human myoglobin differs by at least 35 residues of the approximately 153 residues in each molecule.

**TABLE III**

<table>
<thead>
<tr>
<th>Species</th>
<th>Amino terminal sequence</th>
<th>Carboxyl terminal sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Gly-Leu-Ser-</td>
<td>-Leu-Gly-Phe-Gly-Glu-NH₂</td>
<td>This paper*</td>
</tr>
<tr>
<td>Whale</td>
<td>Val-Ala-Gly-</td>
<td>-Leu-Gly-Tyr-Glu-Glu-Glu-NH₂</td>
<td>(40)</td>
</tr>
<tr>
<td>Horse</td>
<td>Gly-Leu-</td>
<td>-Leu-Asp-Phe-Gly-</td>
<td>(30, 33)</td>
</tr>
<tr>
<td>Seal</td>
<td>Gly-</td>
<td>-</td>
<td>(38)</td>
</tr>
</tbody>
</table>

* Detailed sequence analysis of human myoglobin will be published later.

**SUMMARY**

1. Human metmyoglobin can be resolved chromatographically on DEAE-cellulose into at least four distinct heme protein components.
2. The two chromatographic components that account for 75 to 80% of the total myoglobin of normal human muscle appear to differ from one another only in their heme prosthetic group.
3. Some of the chromatographic components present in minor amounts in human myoglobin appear to differ in their primary structure. The exact differences have not been established at this time.
4. The physical and chemical properties of chromatographi-
ally pure human myoglobin are similar to those reported previously.

5. Certain primary structural differences between human globin and the globin from other species have been shown.

REFERENCES

The Characterization of Adult Human Myoglobin
Gerald T. Perkoff, Robert L. Hill, Douglas M. Brown and Frank H. Tyler