Isolation of Sperm Whale Myoglobin by Low Temperature Fractionation with Ethanol and Metallic Ions*

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SUMMARY

1. A method is described for the isolation and purification of myoglobin from sperm whale skeletal muscle. The aqueous extract is brought to 25% ethanol by volume at −10 to −12° and pH 6.5. A fraction precipitated with lead subacetate is discarded. Myoglobin is precipitated from the supernatant solution at pH 7.20 by adding zinc acetate to a concentration of approximately 10 mM. The precipitate is dissolved in the cold by addition of EDTA, dialyzed against EDTA and water, and rendered salt-free by ion exchange resins. Under favorable conditions the myoglobin is almost entirely in the oxyform.

2. Removal of impurities and conversion to ferrimyoglobin and deoxygenated ferromyoglobin are described according to several procedures. Purity is assayed by chromatography, electrophoresis, spectrophotometric analysis, and immunological analysis.

3. Crystallization of oxymyoglobin is described. Conversion to the ferri form in the crystalline state does not appear to cause disruption of the crystals.

4. Stability of oxymyoglobin prepared by the zinc-ethanol procedure is comparable to that of chromatographically separated preparations. It is much better than that of oxymyoglobin prepared by reduction of ferrimyoglobin by dithionite. The present procedure avoids the rapid autoxidation that occurs during the preparative procedure of Kendrew and Parrish.

5. Absorption and optical rotatory spectra of the highly purified preparations are presented.

6. The microheterogeneity of sperm whale myoglobin described by Edmundson and Hirs is confirmed. Evidence is presented that the different components differ from each other in composition by steps of about the same degree, quite possibly in amide content as suggested by Edmundson and Hirs.

This paper presents a method of isolation of myoglobin from sperm whale muscle by low temperature fractionation with ethanol and metallic salts (4). The method has the advantage of avoiding or minimizing the conversion of oxymyoglobin to ferrimyoglobin which occurs during the salt fractionation procedure described by Kendrew and Parrish (5). This distinction is important, since it is difficult to obtain a stable preparation of oxymyoglobin following reduction of ferrimyoglobin to ferromyoglobin.

Molar extinction coefficients reported here for oxymyoglobin obtained immediately after low temperature fractionation are much higher than any previously reported for the sperm whale protein and are only slightly improved by further purification by ion exchange chromatography. The latter procedure follows the method of Yamazaki, Yokota, and Shikama (6) developed for horse heart myoglobin. The application of a diaphorase reduction method (7) to convert ferrimyoglobin to the reduced, deoxygenated form, ferromyoglobin, is described.

The purification is followed by immunological as well as chromatographic, electrophoretic, and spectrophotometric techniques. The crystallization of oxymyoglobin is described. Microheterogeneity is demonstrated in all preparations and derivative forms studied.

EXPERIMENTAL PROCEDURE

Isolation and Purification of Myoglobin—Frozen sperm whale skeletal muscle supplied by Hvalur (Reykjavik, Iceland) or British Columbia Packers (Vancouver, British Columbia) was partially thawed overnight at 4°, cut into small cubes, and immediately placed in water (about 2 liters per Kg of muscle). The muscle cubes, covered completely with water, were blended for 10 to 15 sec in a Waring Blender at low speed and the suspension was pressed through folded muslin. The extract was

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The general method (4) used here derives from the original observations of J. M. Gillespie (8). Similar conditions were used in an unpublished isolation of human hemoglobin (E. J. Cohn, F. R. N. Gurd, and G. S. Benjamin, 1953).
filtered by suction through coarse filter papers which were changed frequently. All steps above were done at 4°, the maximum temperature employed in the preparative procedures unless otherwise specified. Measurements of pH were made on sufficiently large aliquots of suspension or solution that the working temperature was nearly maintained.

The water extract was adjusted to pH 6.5 ± 0.2 with 0.5 N NaOH, and the temperature of the extract was lowered to 0°. Ethanol (95%), precooled in a Dry Ice-ethanol bath, was slowly added with stirring until the solution was 25% ethanol by volume. During the addition of the ethanol, the temperature was lowered to -10 to -12°. Saturated lead acetate solution (5, 9-11) was added dropwise to a final concentration of 3.4 mM. The suspension was stirred for 1 to 2 hours, then centrifuged at -10 to -12° for about 45 min at 10,000 × g. The precipitate was discarded.

The supernatant was adjusted to pH 7.20 ± 0.20 with 0.5 N NaOH. The Mb2 fraction was precipitated by adding 0.5 mM zinc acetate solution to a final concentration of 10 mM and readjusting the pH to 7.20. After stirring for several hours at -10 to -12° the suspension was centrifuged as above and the supernatant solution was discarded. The precipitate was drained free of supernatant solution. The Mb-zinc precipitate was dissolved at 0-4° with precooled 0.4 M EDTA, pH 7.8, and dialyzed first against 0.02 M EDTA and then exhaustively against water at 4°. All dialysis of Mb solutions was done with Visking cellulose tubing, size 18, which had previously been repeatedly soaked and washed in boiling water. The total yield for the zinc-ethanol procedure was 30 to 35 g per Kg of muscle.

The preparation at this stage contained small quantities of non-myoglobin impurities. At this point purification could be directed either to the preparation of oxyMb by chromatographic separation from traces of ferriMb and non-myoglobin impurities, or to the preparation of ferriMb by an ammonium sulfate fractionation to remove the non-myoglobin impurities and complete oxidation by ferriyanide.

Purification of Oxymyoglobin—The preparation ofMb completely in the oxyMb form was accomplished by ion exchange chromatography on DEAE cellulose by a procedure very similar to that described by Yamazaki et al. (6). Samples used for this separation were freshly prepared by the zinc-ethanol procedure or were preparations that had been stored by freezing the salt-free solutions and maintaining them at -17°.

Columns with bed sizes of 2.0 × 25 cm and 4.25 × 45 cm were packed with previously washed DEAE-cellulose (0.84 meq per g, Schleicher and Schuell) under air pressure of 8 to 10 p.s.i. and equilibrated with 0.005 M Tris buffer, pH 8.4. Samples as large as 0.5 g were placed on the small column.

Preparation of Ferriyanide—To a 6% solution ofMb prepared by the zinc-ethanol procedure, solid (NH4)2SO4 was added to give 50% saturation. The solution was filtered by suction and the precipitate, mainly non-heme proteins, was discarded. Small increments of solid (NH4)2SO4 were added and aliquots of the suspension filtered until the precipitate filtered off was mostly heme protein (about 60% saturation). The ammonium sulfate concentration of the supernatant was then raised to 80% at which point nearly all of the Mb was precipitated and was collected by filtering. The approximate yield per Kg of meat was 25 g.

The precipitate was then dissolved in water to yield about a 5% Mb concentration, and potassium ferricyanide was added in 10 to 20% molar excess to oxidize the remaining ferroMb to ferriMb. The protein solution was then dialyzed against several changes of 0.15 M potassium chloride and then exhaustively against water. Complete deionization of Mb solutions was assured by passage through a Rexyn IRG-501 mixed bed resin (Fisher) or a Dianion column (12) or both.

For many purposes purity at this stage is satisfactory. Further purification was obtained either by molecular sieve chromatography or by carboxymethyl cellulose chromatography. These chromatographic procedures were also used to determine the purity of the Mb preparations not carried through the column purifications.

Molecular Sieve Chromatography—The method of gel filtration chromatography with Sephadex G-50 or G-75 columns was similar to that described by Awad, Cameron, and Kotite (13) for the separation of hemoglobin from Mb. Sephadex G-50 in the bead form (fine) was used in a column with bed dimensions of 2.2 × 115 cm; 0.05 M phosphate, pH 6.6, was used as the eluent. Flow rates were from 5 to 7 ml per hour. The Sephadex was washed free of fines. The columns were packed with an extension tube (14) to avoid zones of fine particles, and equilibrated. It was found preferable to add the samples by first increasing their density with 25% sucrose and then layering on top of the column under the eluent (14). The effluent was monitored by a Gilson UV280 IF monitor or by measuring the absorption at 280 and 409 nm in a Beckman DU spectrophotometer.

Carboxymethyl Cellulose Chromatography—Ion exchange chromatography of ferriMb was carried out on a column of CM-cellulose (0.6 meq per g, Schleicher and Schuell) at 23° with a gradient of phosphate buffer. A column, 1.9 × 24 cm, was packed under 10 to 12 p.s.i. air pressure and equilibrated with 0.01 M potassium phosphate buffer, pH 6.10. The elution gradient was obtained with equal volumes (3 liters) of equilibrating buffer and a 0.01 M potassium phosphate-0.07 M KCl buffer, pH 6.48, connected by a siphon. About 250 mg of ferriMb in 0.01 M phosphate at pH 6.10 were applied to the column. The effluent was collected at the rate of about 100 ml per hour in 10-ml fractions. The absorbance of the fractions was measured at 280 and 409 nm.

Concentration of Dilute Protein Solutions—Dilute solutions of Mb fractions obtained from various types of chromatography were concentrated by pressure dialysis or by dialyzing the solution against 20 to 30% dextran (Grade 60-C, 73,000 average molecular weight, Sigma).

Preparation of Antiserum—New Zealand rabbits (1.5 to 2 Kg initially) were injected with ferriMb mixed with two different adjuvants, the alum-precipitated antigen according to Karush and Marks (15) or commercial complete Freund’s adjuvant (16). The alum-precipitated antigen was prepared by adding

An alternative method with the use of barium acetate instead of zinc at a final concentration of 20 mM also gave satisfactory results.

6 Each time the solution was filtered samples of the supernatant liquid and of the precipitate were each diluted with water and the spectra recorded. The approximate percentage of Mb present was determined by comparing absorbances at the 418 nm peak and the 280 nm peak.

The abbreviations used are: Mb, myoglobin; ferriMb, ferri-myoglobin; ferroMb, ferromyoglobin (deoxygenated); oxyMb, oxymyoglobin; apoMb, apomyoglobin.
an equal volume of 10% alum (aluminum potassium sulfate) to a solution of ferriMb (6 mg per ml) prepared by the zinconethanol method. The pH was maintained near 7 by adding 1 x NaOH simultaneously. The precipitate was centrifuged, washed twice, and resuspended to the original volume of ferriMb with 0.160 NaCl. The final pH was adjusted to 7.5. Each rabbit received about 80 to 100 mg over the course of injections, four times weekly for 4 weeks. The 1st week each received 0.5 ml per injection intravenously in the marginal ear vein. The 2nd, 3rd, and 4th weeks, each received 1, 2, and 3 ml, respectively, by subcutaneous injection in the back. Antibody production was checked by cardiac puncture 7 and 9 days after the last injection, allowed to clot, centrifuged, and pooled. The amounts of precipitating antibodies in the antiserum were determined by the quantitative precipitin method (16) and protein analyses were done by the colormetric procedure of Lowry et al. (17).

**Ouchterlony Technique**—The myoglobin-antimyoglobin reaction was studied by the method of double diffusion in two dimensions according to Ouchterlony (18). Solutions of 0.5% agar (Ionagar, Consolidated Laboratories) in 0.160 NaCl were poured into 6 cm diameter thick-walled Petri dishes (7 ml per dish) and chilled at 4°C. The antiserum was generally placed in a center well and sometimes was allowed to diffuse into the gel for 12 to 24 hours before antigens were placed in the six outer wells. The plates were developed at 4°C and observed for at least 2 weeks.

The approximate amounts of non-myoglobin proteins in different preparations and chromatographic fractions were determined by the Ouchterlony technique. It was found that the different non-myoglobin protein components separated by Sephadex G-75 chromatography each could be detected in Ouchterlony plates in concentrations of 10 μg per ml and above with rabbit antiserum. Mb preparations and fractions to be tested were permitted to react with antiserum in Ouchterlony plates in a series of different concentrations. All antigen volumes and all antiserum volumes applied to the Ouchterlony plates were kept constant. The percentage of non-myoglobin protein was then determined by observing what was the highest concentration of Mb that showed no line of precipitation with the given antiserum other than the myoglobin-antimyoglobin line.

**Polyacrylamide Gel Electrophoresis**—Zone electrophoresis in polyacrylamide gels was done by two different techniques: the vertical gel technique (19, 20) and the disk electrophoresis technique (21). For the former method, the following buffers were used: Tris-EDTA-borate (0.1, 0.0025, and 0.004 M, respectively, pH 8.9 to 9.2); phosphate (ranging from 0.01 to 0.03 M, pH 5.8 to 10.1); acetate (0.1 M, pH 6.0); and phosphate-EDTA (0.02 M phosphate and 0.002 M EDTA, pH 7.5). Voltages ranged from 140 to 400 volts and the current varied from 100 to 230 ma depending on the buffer used.

The technique used in two-dimensional gel electrophoresis was similar to that described by Raymond and Aurrell (22). On completion of the electrophoresis in the first dimension (5% gel in the Tris-EDTA-borate buffer, pH 9.1), a 3- to 5-mm strip was cut out, imbedded in 10% gel, and subjected to electrophoresis with the same buffer in the second dimension. For the disk electrophoresis technique samples were run in 7.5% polyacrylamide gel (Canalco standard gel) at pH 9.5 with glycine-Tris buffer, 0.037 and 0.065 M, respectively. Complete polymerization of the sample without loss of sample could be accomplished by adding a layer of water on top of the sample gel.

A type of "immunoelectrophoresis" was carried out by the disk electrophoresis technique in conjunction with the Ouchterlony technique. Samples were first subjected to electrophoresis in duplicate, then one sample was stained while the other was removed from the tube after electrophoresis and cut into thin cross sections with a razor blade. Each section was placed with a drop of 0.9% NaCl directly into an antigen well of an Ouchterlony plate and the plate was developed as usual.

**Determination of Molar Extinction Coefficients**—The concentrations of ferriMb and apoMb solutions were determined by measuring dry weights of standard deionized solutions. Drying to constant weight was carried out at 107°C. Standard solutions were diluted quantitatively and the absorption spectra determined on a Cary model 14 pm recording spectrophotometer.

Molar extinction coefficients, ε, of ferroMb were determined on samples prepared by three different methods. The first was chemical reduction. Small amounts of solid sodium dithionite were added to standard solutions of ferriMb (approximately 50 μM) in 0.005 M Tris, pH 6.0, which had previously been flushed with nitrogen, and the absorption spectra were recorded.

The second method was an enzymatic reduction which utilized DPNH, methylene blue, and methemoglobin reductase (diaphorase, Calbiochem). This was done according to the procedure described by Benech et al. for hemoglobin (7). A spectrophotometric cell was attached to a Warburg flask with side arms which in turn was connected to a Warburg manometer. FerriMb solution (3 ml) in 0.05 M phosphate buffer, pH 7.3, was placed in the main compartment of the flask while 0.1 ml of methylene blue, 0.1 mm, 0.2 ml of DPNH, 25 mg per ml, and 0.1 ml of diaphorase, 1 mg per ml, all in the same buffer, were placed in the side arm. Argon (Matheson Company, Gold Label purity) bubbled through an alkaline pyrogallol solution was passed through the flask for 2 to 3 min, while the solution was rocked gently. The spectrum of the ferriMb was taken at this point to determine the protein concentration. The contents of the side arm were then tipped into the main compartment and the passage of argon was continued for another 20 min, after which the absorption spectrum of the ferroMb was recorded. This equilibration period was found to be sufficient.

The first two methods could only be used to determine the visible spectrum from about 490 to 700 μm. For the spectrum of ferroMb in the ultraviolet and Soret regions as well as the visible region, however, a third method was used. A sample of oxyMb prepared by separation on a DEAE-cellulose column was deoxygenated by flushing with purified argon for about 2 hours in a similar Warburg flask and the spectrum was then recorded.

For oxyMb, spectral properties were determined by three methods. Dithionite reduction was carried out as described for ferroMb above and then the solution was equilibrated with oxygen. Small amounts of Rexyn IRG-501 mixed bed ion exchange resin (Fisher) were added to remove excess dithionite and any of its oxidation products. Aliquots were then added either directly to the spectrophotometer cells or to given volumes of 1 mM EDTA or 0.05 mM acetate buffers, pH 5.7, previously placed in the cells, and the spectra were promptly recorded. The second method employed the diaphorase reduction. FerriMb solution (3 ml) in 0.05 M phosphate, pH 7.3, was placed in a 1-cm cell and the spectra were recorded. No deaeration was
carried out for any of the solutions. To this mixture was added 0.4 ml of buffer containing DPNH, methylene blue, and diaphorase to give final concentrations of 0.5 mg per ml, 8.3 μM, and 0.04 mg per ml, respectively. The reaction was followed by the increase in absorption at 543 or 581 μM until maximum oxygenation of the Mb was obtained. The absorption of the other maximum was then measured and a scan of this region was made. Corrections were made for the methylene blue absorption by measurements at 662 μM. The third method involved taking the absorption spectra of fractions of oxyMb freed from ferriMb by DEAE-cellulose chromatography. The spectra were measured immediately after elution from the column. The concentrations were determined afterwards by oxidizing the sample with ferricyanide and measuring the absorption at 503 μM (εMax for ferriMb is 9000, below pH 7).

Determination of the percentage of oxyMb in Mb samples was made from measurements at 581.5 μM. After the spectrum of an oxyMb sample was determined (from 700 to 490 μM in 0.02 M phosphate, 2 mM EDTA buffer, pH 0.95), a small crystal of ferriMb was added to convert the sample entirely to ferriMb in order to determine the total Mb concentration.

Preparation of Oxymyoglobin Crystals—OxyMb crystals have been produced directly from zinc-ethanol preparations and also from fractions further purified with DEAE-cellulose columns. From zinc-ethanol preparations, crystals have been grown by adding saturated (NH₄)₂SO₄ (pH values near 5.7 gave best results) to about 3 mM protein solutions. Ratios of volumes of saturated (NH₄)₂SO₄ to protein ranged from 3 to 5.

Crystals were also obtained from oxyMb collected from DEAE-cellulose columns without reconcentration. Concentrations as low as 35 μM were used and crystallization occurred during dialysis against saturated (NH₄)₂SO₄ (pH 6.2).

Optical Rotatory Dispersion Measurements—Optical rotatory dispersion measurements were made on the Bendix Polarmatic instrument (460 3002) by the general technique described previously (23). Generally cells of 1- or 10-mm path length and temperatures of 25° were used. Results reported as effective mean residue rotation, [m]°, and helix contents were calculated directly from [m]°best. The [m]°best values for the samples studied were compared with the values for completely random coil and completely α-helical forms of poly-α-L-glutamic acid, which were taken as -1,700 and -14,200°, respectively (23).

Miscellaneous—Procedures such as pH determination, amino acid analysis, and apoMb preparation have been previously described (24–26). The iron analysis of Mb was done by atomic absorption spectroscopy (27) in which the samples were dried at 105° to constant weight, ashed at 650°, and dissolved with HCl.

RESULTS AND DISCUSSION

Comparison of Fractionation Procedures—The purification of Mb by the zinc-ethanol procedure or the combination of zinc-ethanol plus (NH₄)₂SO₄ fractionation was accomplished much more quickly than by the procedure of Kendrew and Parrish (5).

Purity was assayed by: (a) chromatography on Sephadex, a procedure adapted also to further purification of relatively small quantities of Mb; (b) immunchemical analysis against rabbit antisera produced against whole zinc-ethanol preparations and thus containing antibodies against contaminating proteins; and (c) observation of the ratio of the extinction coefficients at 409 and 280 μM. The removal of impurities was also confirmed by CM- and DEAE-cellulose chromatography and by gel electrophoresis, although these procedures were applied primarily to evaluating the heterogeneity of the Mb itself.

Table 1 summarizes the results of the different fractionation procedures. The contaminating non-myoglobin fractions are described in terms of the total percentage of the preparation that they represent and also the number of such fractions that can be recognized. The approximate yield of Mb in each preparation in grams per kg of muscle processed is given. In the case of column chromatography purification, the yields are given as percentage of the protein applied to the column. The minimum time of preparation is included.

Analysis for Impurities—Chromatographic Components—The purity of the zinc-ethanol preparation was explored by molecular sieve chromatography on Sephadex G-50 and G-75. It was found to contain about 6 to 8% other proteins. For Mb, a typical chromatogram from G-50. The amounts of the colorless proteins (non-heme) found in Fractions I and III were estimated by integrating the area under the 280 μM absorption curve and assuming εMax = 9 for these proteins at 280 μM. This value is similar to that of apoMb, 9.3. Fractions I and III, therefore, contained about 3 and 5%, respectively, of the total protein eluted from the column.

Fraction II, judged by its Soret band to be a heme protein, was shown immunologically to be identical with Mb (discussed later). Considering the effluent volume at which it emerged, it must represent an aggregated form. In this particular zinc-ethanol preparation, the aggregate represents about 1% of the total protein.

Similar but somewhat less complete separations were obtained with Sephadex G-75 than with G-50.

Immunochemo Analysis—The level of contaminating proteins in the zinc-ethanol preparations was also estimated immunochemically and was found to agree with the estimation by Sephadex chromatography. Rabbit antisera against the whole zinc-ethanol preparations contained antibodies against the components of Sephadex G-50 fractions I and III, as well as against Mb.

Ouchterlony plates on which zinc-ethanol preparations were allowed to react with undiluted antiserum under conditions of relatively high antigen concentrations (1.0 or 2.0 mg per ml).

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### Table I

<table>
<thead>
<tr>
<th>Type of preparation</th>
<th>Total yield</th>
<th>No. of components</th>
<th>Minimum time for preparation</th>
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<tr>
<td>Aqueous muscle extract</td>
<td>20 to 30</td>
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<tr>
<td>Zinc-ethanol</td>
<td>6 to 8</td>
<td>2</td>
<td>35 to 35°</td>
</tr>
<tr>
<td>Zinc-ethanol + (NH₄)₂SO₄</td>
<td>1</td>
<td>2</td>
<td>25°</td>
</tr>
<tr>
<td>Kendrew and Parrish (5)</td>
<td>5</td>
<td>2</td>
<td>20 to 25°</td>
</tr>
<tr>
<td>Zinc-ethanol + Sephadex C</td>
<td>&lt;0.5</td>
<td>70 to 75°</td>
<td>24°</td>
</tr>
<tr>
<td>Zinc-ethanol + DEAE-cellulose</td>
<td>&lt;0.5</td>
<td>70 to 75°</td>
<td>24°</td>
</tr>
</tbody>
</table>

* Grams of myoglobin per kg of muscle.  
† Percentage of total protein applied to column.
Isolation of Sperm
lines, the amounts of the minor components were estimated.
the antigen wells of an Ouchterlony plate and observing what
minimum concentration was necessary to produce any minor
detectable lines of antigen-antibody complex with antiserum.
dex fractions, I and III, in concentration of 10 pg per ml produced
produced by components of G-50 Fraction I. Each of the Sepha-
dexles. The major line, nearest the antibody well, was due to
chromatography. The antigen causing the second line was
identified on Ouchterlony plates as the main component of G-50
showed three or sometimes four lines of antigen-antibody com-
plexes. The major line, nearest the antibody well, was due to
the myoglobin antimyoglobin complex. The others indicated
two or three minor components as also shown by Sephadex G-50
chromatography. The antigen causing the second line was
identified on Ouchterlony plates as the main component of G-50
Fraction III. The third line (and fourth when present) was
produced by components of G-50 Fraction I. Each of the Sepha-
dex fractions, I and III, in concentration of 10 µg per ml produced
detectable lines of antigen-antibody complex with antisemur.
By applying a series of concentrations of a given Mb sample to
the antigen wells of an Ouchterlony plate and observing what
minimum concentration was necessary to produce any minor
lines, the amounts of the minor components were estimated.
With zinc-ethanol preparations, Line 3 (G-50, Fraction I) was
barely visible at myoglobin concentrations of 500 mg per ml and
was absent at lower concentrations. Line 2 (G-50, Fraction III)
was barely visible at 200 mg and was absent below. These
observations establish the amounts of these components at about
2 and 5%, respectively. Zinc-ethanol preparations further
purified by (NH₄)₂SO₄ fractionation (discussed later) were shown
to contain about 1 to 1.5% of non-myoglobin components by
this technique.
Spectrophotometric Analysis for Impurities—Most routine
tests of purity on the ratio of the 409 to 280 nm peaks. The extinctions for
these wave lengths for solutions purified by molecular sieve or
ion exchange chromatography repeatedly were found to be
16.6 × 10⁴ and 3.16 × 10⁴ ± 0.1 × 10⁴, respectively, at pH
values below 7, giving a ratio of 5.25 ± 0.05.
Good agreement has been found among all three of these
tests of purity.
Methods for Further Purification—For most purposes it is
desirable to use Mb samples with higher degrees of purity than
obtained by the zinc-ethanol procedure alone. The most effi-
cient method used for increasing the purity of large amounts of
zinc-ethanol-prepared myoglobin (e.g. 30 g) was the (NH₄)₂SO₄
fractionation (see "Experimental Procedure"). This step
lowered the amounts of non-myoglobin proteins from 6 to 8% to
about 1%. With a sample of ferriMb purified by zinc-ethanol
and (NH₄)₂SO₄ fractionations, Line 3 did not appear even at
concentrations as high as 2.0 mg per ml, indicating that this
component (G-50, Fraction I) was present in amounts less than
0.5%. Line 2 appeared very weakly at 1.0 mg per ml and was
absent at 0.5 mg per ml of myoglobin concentrations, indicating
that this component was present in amounts of about 1%. The
percentage of iron found in this preparation was 0.310 ± 0.005
as compared to the theoretical value of 0.3134% based on a
molecular weight of 17,816 (28). Amino acid analyses of this
preparation agreed very closely with those reported by Ed-
mundson and Hirs (28).
In order to obtain myoglobin complex with contaminating
protein levels of less than 1%, Sephadex G-50 or G-75 chroma-
tography was used (Fig. 1). The first few milliliters of Fraction
IV (Mb fraction) contained trace amounts of Fraction III.
However, at 205 ml, well before the peak of the fraction (210
to 215 ml), no trace of Fraction III could be detected on Ouch-
terlony plates at Mb concentrations of 2.0 mg per ml. This
indicates that there was less than 0.5% contamination before
the peak was reached and that the fraction consisting of the
protein collected after 205 ml would contain very much less than
0.5% of other proteins.
Aggregation of Mb, detected after the zinc-ethanol prepa-
ration at a level of about 1% (Fig. 1), was found to be greater
after subsequent (NH₄)₂SO₄ fractionation. An example of
Sephadex chromatography of zinc-ethanol-prepared ferriMb
further purified by an (NH₄)₂SO₄ fractionation is illustrated in
Fig. 2. Testing this first fraction on Ouchterlony plates
revealed that this component again is Mb and therefore must be
an aggregate. In Fig. 2, the aggregated protein amounted to
about 5% of the total protein. The spectrum indicated that
extensive disruption of the Mb structure in this fraction was
unlikely. During crystallization of Mb from concentrated
(NH₄)₂SO₄ aggregated noncrystalline material is observed,
quite probably formed by surface denaturation. The aggregates
favored in the present procedure are readily removed by molecu-
lar sieve chromatography.

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It was assumed that the zinc-ethanol preparation contained
no contaminating proteins which were nonimmunogenic in the
rabbit. No other contaminating proteins were found by repeated
gel electrophoresis.
Ion exchange chromatography on CM- and DEAE-cellulose, which has been previously used for the isolation of other myoglobins (29, 30) was also very satisfactory for the removal of impurities from zinc-ethanol preparations of sperm whale Mb. Here, however, the main application of CM-cellulose was the separation of the heterogeneous Mb fractions, while DEAE-cellulose was mainly used for separation of oxyMb from traces of ferriMb.

**Isolation and Stability of Oxymyoglobin**—The zinc-ethanol procedure as described produced oxyMb preparations virtually free of ferriMb. Other preparations in which the ground muscle-water extract mixture was allowed to stir overnight yielded preparations containing 80 to 90% oxyMb. By homogenizing the muscle in a Waring Blender and allowing it to stir for only about 30 min, according to the observations reported by Yamazaki et al. (6) for preparing horse heart oxyMb, it was possible to obtain sperm whale oxyMb almost free of any ferriMb (97% oxyMb) directly from the zinc-ethanol method without the use of ion exchange separation. The sperm whale muscle was frozen in Dry Ice immediately after removal from the whale and shipped with precautions to avoid thawing. Starting with 1 Kg of muscle, it was possible to prepare 30 to 35 g of oxyMb by this procedure in 14 hours, excluding dialysis time.

The DEAE-cellulose method of Yamazaki et al. (6) was suitable for separating oxyMb prepared by the zinc-ethanol procedure from any traces of ferriMb and for removing non-myoglobin impurities. Fig. 3 shows the spectrum of oxyMb prepared by DEAE-cellulose chromatography, compared with that of ferriMb.

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**Fig. 2.** Sephadex G-75 chromatogram of ferrimyoglobin purified by the zinc-ethanol procedure and (NH₄)₂SO₄ fractionation. Ferrimyoglobin (2 ml, 70 mg) in 0.05 M NaCl, pH 7.5, was added to a column (1.9 x 150 cm, bed dimensions) previously equilibrated and eluted with 0.05 M NaCl at a flow rate of about 30 ml per hour. Fraction II represents the main myoglobin fraction, while Fraction I was shown in combination with its reaction with antimyoglobin serum to be an aggregate of myoglobin (see text). Absorbance at 409 μm, ———, and absorbance at 280 μm, ......

**Fig. 3.** Absorption spectra of ferrimyoglobin, solid line, and oxymyoglobin, dashed line. The ferrimyoglobin was isolated by the zinc-ethanol procedure, oxidized by ferricyanide, and was further purified by Sephadex G-50 chromatography (see Fig. 1). The oxymyoglobin was also isolated by the zinc-ethanol procedure and was freed from traces of ferrimyoglobin and other proteins by DEAE-cellulose chromatography (see text).
Isolation of Sperm Whale Myoglobin

Table II

Molar extinction coefficients of maxima for derivatives of sperm whale myoglobin

In Column 2 $e_{\text{max}}$ values are shown for derivatives of myoglobin isolated from the muscle by the zinc-ethanol procedure and DEAE-cellulose chromatography, in the native (ferro) state. These values were measured at pH 7.4 in 0.005 M Tris buffer. In Columns 3 to 5 $e_{\text{max}}$ values are shown for these derivatives which were produced from ferrimyoglobin by enzymatic and chemical reductions, respectively (see “Experimental Procedure”).

<table>
<thead>
<tr>
<th>1. Wave lengths of maxima</th>
<th>2. Isolated in ferro state</th>
<th>3. Reduced enzymatically (diaphorase)</th>
<th>4. Reduced chemically (dithionite)</th>
<th>5. Samejima and Yang (31)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$e_{\text{max}}$</td>
<td>$e_{\text{max}}$</td>
<td>$e_{\text{max}}$</td>
<td>$e_{\text{max}}$</td>
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<tr>
<td>Oxymyoglobin</td>
<td>581</td>
<td>1.46</td>
<td>1.37</td>
<td>1.30</td>
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<tr>
<td></td>
<td>543</td>
<td>1.36</td>
<td>1.30</td>
<td>1.26</td>
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<td>418</td>
<td>12.8</td>
<td>11.9</td>
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<td>348</td>
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<td></td>
<td>286</td>
<td>3.66</td>
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<td>Ferromyoglobin</td>
<td>550</td>
<td>1.20</td>
<td>1.18</td>
<td>1.20</td>
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<td></td>
<td>434</td>
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<td></td>
<td>278</td>
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<td>3.50</td>
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<tr>
<td>Carboxyferromyoglobin</td>
<td>579</td>
<td>1.22</td>
<td>1.23</td>
<td></td>
</tr>
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<td></td>
<td>542</td>
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<td></td>
<td>246</td>
<td>3.70</td>
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Table II shows the extinction coefficients of Mb obtained by isolation of the protein in the ferro state and by enzymatic (diaphorase) and chemical (dithionite) reduction.

Previous methods of Mb preparation left the product mostly in the oxidized (ferri) state so that any experiments on ferroMb or oxyMb required the reduction of ferriMb by a chemical reducing agent. The most commonly used of these has been dithionite. Molar extinction coefficients ($e$) obtained for oxyMb by dithionite reduction have been thought to be too low because of rapid autoxidation. These values for maxima in the visible region for ferroMb and oxyMb determined in our laboratory by dithionite reduction agree within 1% of the values reported by Samejima and Yang (31). Nevertheless, the extinction coefficients for the 581 and 543 nm peaks for oxyMb isolated by DEAE-cellulose chromatography (Table II) were much higher than those obtained for oxyMb prepared by dithionite reduction. Furthermore, the dithionite absorption has made measurements in the Soret and ultraviolet regions at best very difficult. OxyMb prepared by enzymatic reduction of ferriMb with diaphorase and subsequent oxygenation gave higher extinction coefficients at 581 and 543 nm than the dithionite reduction, but not as high as those for native oxyMb.

The oxygenated form in the presence of the mixture of the methylene blue and DPNH is not stable, presumably because of reversal of the reaction due to some excess of DPNH.

OxyMb produced by dithionite reduction and subsequent oxygenation has been found to autoxidize to ferriMb much more rapidly than oxyMb isolated directly from the muscle. At room temperature samples of oxyMb produced by dithionite reduction have almost completely returned to ferriMb (90% or more) in 16 hours. Samples of oxyMb isolated directly from the muscle by the zinc-ethanol method and DEAE-cellulose chromatography and stored at 4°C for 48 hours have been found to be over 98% in the oxyMb form. After 24 hours the spectra of these samples were identical with oxyMb spectra obtained immediately after elution from the column. This behavior corresponds very well to the stability of horse heart oxyMb isolated by this procedure by Shikama (32) and Yamazaki et al. (6). The zinc-ethanol procedure applied alone has produced samples which are 97% oxyMb and after 10 days at 4°C have been found still to contain 99% of the oxygenated form.

The rate of autoxidation of oxyMb has been found to be greatly increased by Cu(II) ions but is unaffected by Pb(II), Ba(II), and Ca(II) (2, 33). Although Cu(II) ions oxidize the oxyMb under conditions such that the metal ions do not denature the protein, the same is not true for Zn(II). An increase in autoxidation would be expected under conditions in which denaturation occurs. The precipitation with Zn(II) in the specific metal concentration would normally be considered high enough to promote denaturation (33-35). However, at low temperatures (here -10 to -12°C) Zn(II) ion denaturation is strongly inhibited (38), and no evidence of it has been observed in these preparations.

The basis of the relatively low rate of autoxidation of Mb in the present type of preparation compared with the previous method (5) is unknown. Short exposure in the aqueous muscle extract, the low temperature during processing, or the removal of some organic or inorganic impurity may all play a role. The removal of heme protein that is not myoglobin by the present preparation method or by DEAE-cellulose chromatography applied directly to the muscle extract or to horse heart material (6, 32) may be important. The present preparation may be convenient for studying the complicated processes involved in autoxidation (38).

Absorption Spectra of FerroMb and CarboxyferroMb—The spectra for ferroMb determined by the three different methods were in good agreement. Fig. 4 shows the absorption spectrum of ferroMb produced by deoxygenating oxyMb isolated by DEAE-cellulose chromatography with argon. The ultraviolet and Soret regions of the spectrum were obtained only by the deoxygenation procedure because of the interference caused by the absorption of dithionite or DPNH and enzyme.

The carboxyferroMb absorption spectra obtained by the displacement by CO of the molecular oxygen from DEAE-cellulose-fractionated oxyMb agrees very closely with the spectrum obtained by exposure to CO following dithionite reduction (31). CarboxyferroMb appears to be much more stable to autoxidation than oxyMb presumably because of the much stronger binding of the CO ligand and perhaps because the 6th position of the heme iron must be available in order that oxidation to the ferri form may occur.

Crystalization of Oxymyoglobin—Solutions of zinc-ethanol-prepared oxyMb have been crystallized from (NH₄)₂SO₄. Crystals in the range of 0.1 to 0.3 mm in length were obtained. The crystals were red in comparison to the brown ferriMb crystals. They underwent autoxidation to the ferri form or could be oxidized with ferricyanide with no visible changes in the form of each individual crystal. Attempts to grow larger crystals have
so far met with at least partial autoxidation before the crystals reached the desired size.

Optical Rotatory Dispersion—The optical rotatory dispersion of myoglobin has been discussed previously (37–39), most recently by Harrison and Blout (40), Samejima and Yang (31), and Breslow et al. (23). Samples of ferriMb purified by the zinc-ethanol procedure alone, and samples which then had been further purified by the (NH₄)₂SO₄ fractionation, have repeatedly given \([\rho']_{233}\) values in the range of −8500 to −8800 degrees. Values for samples purified by Sephadex or CM-cellulose chromatography, however, have consistently fallen in the range from −8800 to −9000 degrees. Fig. 5 shows the optical rotatory dispersion of ferriMb purified by Sephadex G-50 chromatography.

Values of \([\rho']_{233}\) for oxyMb isolated by DEAE-cellulose chromatography have been found to fall within the range of values obtained for ferriMb. This observation is in agreement with the optical rotatory dispersion results for oxyMb prepared by dithionite reduction by Samejima and Yang (31). They also found comparable values for ferroMb, carboxyferroMb, and cyanoferrimB. Similar results have been reported for hydroxyl and azide derivatives of ferriMb (23).

Microheterogeneity of Sperm Whale Myoglobin—The heterogeneity of sperm whale Mb described by Edmundson and Hirs (28, 41) was again demonstrated by CM-cellulose chromatography and gel electrophoresis. A CM-cellulose fractionation of ferriMb is shown in Fig. 6. The approximate percentages of total protein were determined for each fraction by estimating the areas under the 280 m\(\mu\) absorption curves. These were found to be 2, 6, 17, and 75% for Fractions I, II, III, and IV, respectively. A small amount of non-heme protein emerged between 70 and 140 ml. These results are similar to those obtained with carboxyferroMb on IRC-50 resin by Edmundson and Hirs (28, 41). The early non-heme protein fraction was shown on Ouchterlony plates to be identical with Sephadex G-50 Fraction I (Fig. 1). CM-cellulose Fractions II, III, and IV reacted with antimyoglobin serum on Ouchterlony plates, all forming single lines of identity with each other. Fraction I was not tested. No attempt has yet been made to show quantitative differences in the reaction of these fractions with antisera, such as by quantitative precipitin or complement fixation techniques. The absorption spectra of Fractions II, III, and IV are very similar; the ratios of absorption at 409 m\(\mu\) to 280 m\(\mu\) for Fractions III and IV are identical within experimental error. The optical rotatory dispersion curves and visible and ultraviolet spectra of Fraction IV are indistinguishable from those for ferriMb preparations containing all components. Edmundson and Hirs (41) report that the amino acid analyses of the four fractions are identical except for the first fraction which in their separation was contaminated with non-heme protein.

7 CM-cellulose fractionation indicated the possibility of a fifth myoglobin fraction preceding Fraction I. However, the absorptions of the tubes in this region were too small to be conclusive. The presence of a fifth fraction, however, has been shown conclusively by electrophoresis (discussed later).
The disk and vertical polyacrylamide gel electrophoresis techniques routinely showed four or occasionally five ferriMb bands depending on the amount of sample applied. The fifth electrophoretic Mb band has been definitely shown to be present but constitutes such a small percentage of the total protein that it is generally not visible (Fig. 7). In the DEAE-cellulose fractionation of ferriMb and oxyMb, the first fraction was ferriMb corresponding to CM-cellulose Fraction IV, with small amounts of III and II, (Fig. 7D), and the second was oxyMb corresponding to the same CM-cellulose fraction. After the buffer change to 0.05 M Tris the remainder of the protein came off the column without being fractionated. This last fraction was converted completely to ferriMb, concentrated, and run on gel electrophoresis (Fig. 7E). The pattern clearly shows the absence of the major component, but the other four Mb fractions are all present. All five electrophoretic fractions of ferriMb...
reacted with antimonyglobin serum in Ouehterlony plates, forming lines of identity with each other, as was also observed for the CM-cellulose fractions. Furthermore, CM-cellulose Fractions IV and III were shown to have mobilities identical with the slowest and next to slowest electrophoretic fractions, respectively.

The disk electrophoretic pattern of ferriMb prepared by the procedure described by Kendrew and Parrish (5) were indistinguishable from those of the cine-ethanol preparation. This and the chromatographic separations which have been done on both types of preparations indicate that these fractions are not artifacts produced by a preparatory method. A sample of carboxyferriMb components IV + V from IRC-50 chromatography (41), a gift from Dr. Allen Edmundson, was converted to ferriMb with potassium ferricyanide and subjected to disk electrophoresis. This fraction migrated identically with CM-cellulose Fraction IV, the major (and slowest) electrophoretic band.

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Re-electrophoresis of the two slowest electrophoretic bands on disk electrophoresis demonstrated that each retained the same mobility on the second run and that each still formed a single band. The same observation was obtained in a different manner with the vertical gel technique. When Mb fractions separated by an initial electrophoresis were cut out in vertical strips, imbedded horizontally in fresh gel, and subjected to electrophoresis in the second dimension (42), the different fractions appeared as single spots on a diagonal line (Fig. 8). The appearance of single spots in each column after electrophoresis in the second dimension clearly indicates that the minor fractions are not formed from the major fraction by some type of transformation. The appearance of a straight diagonal line rather than a curved line when the second dimension was run in a more concentrated gel indicates that these minor fractions do not result from aggregation (42).

Cyanoferrilm showed the same electrophoretic pattern as native ferriMb (Fig. 7B). The mobilities of individual components matched closely the corresponding band of ferriMb except that the cyanoferrilm bands were slightly faster. At the pH at which the comparison was made, pH 9.2, the proton release from the iron-bound water (pK 8.90, see Reference 25) will result in the ferriMb having about 0.67 of an additional negative charge. In the case of the cyano derivative, virtually all of the molecules will have an additional full unit of negative charge. The corresponding bands of the two derivatives would be expected to differ, on the average, by approximately one-third of a charge unit. Assuming that the Mb components differ by unit steps in net charge, the expected influence of the cyanide ion on electrophoresis of ferriMb preparations obtained by T. E. Hugli after short exposure to a-bromopropionate. This reagent attacks much fewer histidine residues than bromoacetate (43). Under certain conditions, to be discussed elsewhere, unitary charge differences between reaction products are to be expected. The observed shifts in mobility match well with those presented here for the various Mb derivatives (Fig. 7). These observations support the thesis that the Mb components differ by steps of one charge, such as might be explained by unitary steps in amide content.

The results with cyanoferrilm and apoMb mentioned above do not contribute any clarification to the complex phenomena that Perkoff et al. (44) found on DEAE-cellulose chromatography and starch gel electrophoresis of human Mb. None of our results showed any indication of an anomaly which might be explained by a transformation involving the ionization of the heme-bound water molecule.

Electrophoresis of guanidinated ferriMb (45) also produced the same pattern as native ferriMb (Fig. 7C). The mobility of each band in this case, however, was slower than the corresponding ferriMb band as a result of the increase of pK of the lysine side chains (ε-amino, 10.6) to several pH units higher in the resulting homoarginine residue. Acid-denatured Mb (exposed to pH 3.0 overnight) was rapidly adjusted to pH 7 and the protein which remained soluble was run in electrophoresis at pH 9.2 and again the same four bands appeared (not shown). The mobility of each band, however, was somewhat slower (and more diffuse) than for native Mb. This could have been due to the failure of the polypeptide chain to refold into the same compact structure as the native protein (25), and possibly reflected some persisting aggregation. It is important to note that in this case, where the conformation of these myoglobin fractions had been altered, the fractions still are differentiated by electrophoresis.

The basis for the differences between each of these five Mb components is not altogether known. In view of all the evidence shown above, it is difficult to conceive of any explanation except that the differences reside in amino acid composition or sequence or both. A likely explanation for this heterogeneity was suggested by Edmundson and Hirs (41) that the differences may lie in variations of total amide content for the glutamic and aspartic residues.

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REFERENCES
Isolation of Sperm Whale Myoglobin by Low Temperature Fractionation with Ethanol and Metallic Ions


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