The electrophoretic inhomogeneity of crystalline horse myoglobin was first observed by Theorell and Ehrenberg (1). Repeated recrystallization failed to eliminate the accompanying material and homogeneous myoglobin could only be achieved by the use of preparative electrophoresis. Theorell and de Duve (2) also noticed a colored substance in preparations of human myoglobin which could be separated from the myoglobin by electrophoresis, but the material was not studied further. While studying the acid cleavage of hemoproteins, crystalline horse myoglobin was found to behave as if it were a mixture of three substances (3); more recently, it was found that at pH 8 three colored components could be detected electrophoretically in samples of crystalline horse myoglobin. These three components appear to be the three substances detected during acid cleavage of myoglobin. These studies have now been extended to beef myoglobin in which it has been found that this myoglobin is also inhomogeneous when crystallized by salting out with ammonium sulfate.

EXPERIMENTAL

Preparation of Myoglobin—The isolation procedure used was similar to that of Roche et al. (4). However, a brief outline of the fractionation procedure will be presented in order to indicate modifications of their method. This method differs from Theorell's original procedure (5) in that the lead acetate step was eliminated.

Defatted beef skeletal muscle was frozen in dry ice and pulverized. This material was extracted twice with an equal weight of water at pH 6.7. The pH was maintained at this value during the remainder of the isolation. All procedures were carried out at 5° ± 3°. Solid ammonium sulfate was added to 85 per cent saturation and the precipitate discarded. A crude myoglobin precipitate was then obtained by completely saturating the remaining solution with ammonium sulfate. The precipitate was dis-
HOMOGENEITY STUDIES

Solved in water to give a myoglobin concentration of approximately 20 mg. per ml. and solid ammonium sulfate was added to 80 per cent saturation. A saturated solution of ammonium sulfate was then added dropwise to the point of incipient cloudiness. If after standing 12 hours the precipitate was amorphous, it was removed and dropwise addition of ammonium sulfate solution was continued until another precipitate formed. This process was repeated until crystalline material was obtained. Usually three or four amorphous precipitates formed before the myoglobin crystallized. The amorphous material contained a colorless protein together with some myoglobin. Finally the myoglobin, now in the met-myoglobin form, was twice recrystallized from ammonium sulfate. Photo-

![Fig. 1](image-url)

**Fig. 1.** (a), crystals of beef metmyoglobin (× 440) produced by slow crystallization (2 days) from (NH₄)₂SO₄ solution; (b), the pattern (× 960) obtained when crystallization was rapid (8 hours).

Micrographs of the crystals are shown in Fig. 1. Slow crystallization (2 days) produced plates, while needles formed if the process was rapid (8 hours). To our knowledge only the former pattern has been reported (4, 6). The iron content was 0.32 per cent, which is in agreement with the value arrived at by Bowen (7) in his thorough study of horse myoglobin.

Horse myoglobin was prepared by the above method from horse skeletal muscle. Crystallization was much simpler, since the colorless protein that interfered with the crystallization of beef myoglobin was not encountered. The iron content was 0.32 per cent.

Crystallization of pork myoglobin was attempted, but accompanying proteins could not be separated by the ammonium sulfate procedure. At least four other proteins precipitated with the myoglobin in the ammonium sulfate saturation range of 85 to 100 per cent. The contaminating proteins were detected by electrophoresis.

*Electrophoretic and Ultracentrifugal Analyses—Electrophoresis was car-*
ried out with a Pearson instrument. Photographs were obtained by the Longsworth scanning method with infra-red sensitive film, a red filter, and a tungsten lamp light source. Phosphate buffer, pH 8, \( \Gamma/2 = 0.1 \), and a migration time of 180 minutes at 20 ma. were employed in all the experiments. Mobilities were calculated from the descending boundaries and were corrected to 0°.

Paper electrophoresis was performed at 5° with Munktell No. 20 paper. Veronal buffer, pH 8.6, \( \Gamma/2 = 0.05 \), was found to give the best results.

Sedimentation studies were carried out with a Spinco model E analytical ultracentrifuge. Phosphate buffer, pH 8, \( \Gamma/2 = 0.1 \), together with 1 per cent NaCl, was used in all experiments. The myoglobin and hemoglobin samples were run with a rotor speed of 59,780 r.p.m. The sedimentation constants were corrected to a water basis at 20°.

Acid Cleavage Curves—The acid cleavage procedure involves spectrophotometric determination of the amount of hemin cleaved after addition of a definite amount of myoglobin of known pH to acetone. The time elapsing from the addition of acid to the reading of the optical density was 6 minutes. The amount of hemin cleaved was calculated from the optical density readings and these values were plotted against the pH of the solution. This method has been described previously (3).

RESULTS AND DISCUSSION

Electrophoretic Inhomogeneity—Two distinct components were detected electrophoretically in the crystalline beef myoglobin preparation (Fig. 2, a). With the phosphate buffer used the mobility of the main myoglobin boundary was \(-1.4 \times 10^{-5} \ \text{cm}^2 \ \text{sec}^{-1} \ \text{volt}^{-1}\), while the accompanying substance (designated Substance A), which amounted to 15 to 20 per cent of the protein in the mixture, migrated with a mobility of \(-2 \times 10^{-5} \ \text{cm}^2 \ \text{sec}^{-1} \ \text{volt}^{-1}\). The electrophoretic mobilities are summarized in Table I. Numerous recrystallizations did not remove this faster migrating substance. A very small peak (less than 1 per cent) could be seen approxi-
mately half an hour after the current was turned on, but at the end of the experiment (3 hours) the peak had flattened out to such an extent that it did not appear on the photographic plate. The mobility of this small component (designated Substance B) was estimated roughly to be $-2.6 \times 10^{-6}$ cm.$^2$ sec.$^{-1}$ volt$^{-1}$. This substance corresponds to the third component noted in crystalline horse myoglobin preparations (3). An electrophoretic diagram of horse myoglobin is shown in Fig. 2, b for comparison. The relative concentrations of Substances A and B were greater in the horse hemoprotein sample, and also the degree of separation of the components was greater in horse myoglobin than in beef myoglobin. Theorell's original procedure, which makes use of lead acetate for removal of extraneous proteinaceous material, failed to eliminate the faster migrating substances. Crystalline myoglobin was isolated from four completely different samples of beef skeletal muscle and in all preparations Substance A was present. The concentration of Substance A in these samples fell within the range of 15 to 20 per cent of the protein in the crystalline material. This variation may be similar to the apparently normal variation of myoglobin in beef which was reported in Paper I of this series (8).

Properties of Substance A—It is believed that Substance A is not an artifact produced by denaturation of the myoglobin, since the isolation procedure was kept as mild as possible. The temperature never exceeded $5^\circ \pm 3^\circ$, nor did the pH vary more than 0.5 unit from neutrality before it was readjusted to pH 6.7 and only ammonium sulfate fractionation was employed.

A sample of Substance A was obtained for spectrophotometric study by subjecting a 2 per cent solution of the myoglobin preparation to elec-

<table>
<thead>
<tr>
<th>Solution analyzed</th>
<th>Sedimentation constant $(s_{20W}) \times 10^{4*}$</th>
<th>Electrophoretic mobility $\times 10^{\dagger}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myoglobin preparation</td>
<td>1.78</td>
<td>-1.4</td>
</tr>
<tr>
<td>80% myoglobin and 20% hemoglobin</td>
<td>1.79</td>
<td></td>
</tr>
<tr>
<td>50% “ “ 50% “</td>
<td>1.81</td>
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</tr>
<tr>
<td>Substance A</td>
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<td>-2.0</td>
</tr>
<tr>
<td>“ R</td>
<td></td>
<td>-2.6</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>4.3</td>
<td>-1.1</td>
</tr>
</tbody>
</table>

* Phosphate buffer, pH 8, $\Gamma/2 = 0.1$, plus 1 per cent NaCl.
† Phosphate buffer, pH 8, $\Gamma/2 = 0.1$. 

Table I

**Sedimentation Constants and Electrophoretic Mobilities of Hemoglobin and Myoglobin Samples**
phoresis for 10 hours. At the end of that time Substance A had completely separated from the main myoglobin boundary and a portion of this faster migrating material was removed from the cell. This sample of course was mixed with some of Substance B which was present in such low concentration that it could not be seen in the electrophoretic diagram. The light absorption spectrum of Substance A, together with the associated small contamination from Substance B, was very similar to that of metmyoglobin. When treated with pyridine, NaOH, and reducing agent, the reduced pyridine hemochromogen spectrum characteristic of hemin was formed. Substance A, therefore, behaves as if it were a hemoprotein. From acid cleavage data (see below) the heme-protein linkages appear to be different in Substance A and myoglobin.

The physiological significance of Substance A, such as its rôle as a respiratory pigment or enzyme, could not be determined because of the lack of sufficient quantity of the substance. Further study of the chemical and physiological nature of the compound will help elucidate its function relative to other cell constituents.

Attempts were made to obtain larger quantities of Substance A by means of paper and starch electrophoresis. Myoglobin and hemoglobin could be easily separated, but the best resolution of Substance A and myoglobin that could be obtained was the formation of a doublet zone. The possibility that Substance A might be hemoglobin which had not been completely removed during fractionation was considered. This was found not to be the case, however. Crystalline, electrophoretically homogeneous beef oxyhemoglobin was prepared (9), converted to methemoglobin, and added to a portion of the myoglobin preparation. The concentration of Substance A or B did not increase, while, in fact, the hemoglobin appeared as a third slower moving peak (Fig. 2, c). The mobility of the hemoglobin alone or in the presence of myoglobin was $-1.1 \times 10^{-5}$ cm.$^2$ sec.$^{-1}$ volt.$^{-1}$, which is considerably less than any of the myoglobin components (Table I).

**Ultracentrifugal Studies with Crystalline Myoglobin**—The crystalline beef myoglobin was subjected to ultracentrifugal analysis and, although electrophoretically inhomogeneous, the preparation exhibited only one peak during sedimentation (Fig. 3, a). The sedimentation constant, $s_{20}$, for the boundary was 1.78 S ($S = 10^{-12}$ cm. per second per unit field of force). The sedimentation constants are summarized in Table I. The presence of only one peak indicated that either Substance A was of similar molecular weight (assuming similar molecular shape) or that an association complex was formed with the myoglobin which prevented resolution. This problem has not been resolved, but some interesting data on protein association were obtained while attempting to arrive at an answer.
Since Substance A appeared to be a hemoprotein, it was of importance to determine the degree of separation of hemoglobin and myoglobin in the ultracentrifuge. The sedimentation constant of hemoglobin in the buffer system used was first determined and was found to be 4.3 S (Fig. 3, d). Then a mixture of 20 per cent hemoglobin and 80 per cent myoglobin (total protein concentration was 5 mg. per ml.) was subjected to ultracentrifugal analysis. Only one boundary with a sedimentation constant of 1.79 S was apparent (Fig. 3, b). A solution which contained equal amounts of hemoglobin and myoglobin (total protein concentration was again 5 mg. per ml.) was also tested. Again, only one boundary was noted (Fig. 3, c). The $s_{20}$ value was 1.81 S. Although two peaks were not found, it can be seen that the sedimentation boundary of hemoglobin or myoglobin alone is much sharper and less diffuse than the boundary of a mixture of the two substances.

It is apparent that, even with a sedimentation constant difference of over 2, myoglobin and hemoglobin cannot be separated by velocity sedimentation in the buffer system employed in these studies. By calculation it was found that, under the conditions used, the two materials should have separated by a distance of nearly 0.6 cm. on the photographic plate.

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**Fig. 3.** Velocity sedimentation photographs of (a) beef myoglobin, (b) a mixture of 80 per cent myoglobin and 20 per cent hemoglobin, (c) a mixture of 50 per cent each of myoglobin and hemoglobin, and (d) beef hemoglobin. (a), (b), and (c) taken 128 minutes after attaining maximal rotor speed; (d) after 96 minutes.
Therefore, it cannot be assumed that Substance A has a molecular weight similar to that of myoglobin. These results again point out the fact that ultracentrifugal homogeneity alone cannot be considered sufficient evidence for assumption of complete homogeneity. The work of Longsworth (10) with ovalbumin has clearly demonstrated this. Ovalbumin was homogeneous by ultracentrifugation, but more than one component was detected by electrophoresis.

It was surprising to find that the sedimentation constants for the mixtures of hemoglobin and myoglobin were very near the value obtained with the myoglobin preparation alone (Table I). The presence of myoglobin de-

- Fig. 4. Acid cleavage of beef metmyoglobin and the logarithmic plot of the data

itely prevented the hemoglobin from sedimenting at its normal rate. If an association complex were assumed to be formed, it is difficult to understand why the mixture of half myoglobin and half hemoglobin sedimented at a rate nearer to the value for myoglobin rather than to some intermediate value. Although it is only speculation, the data could be interpreted to mean that the hemoglobin dissociated into 4 units of equal size when mixed with myoglobin. Since the molecular weight of hemoglobin is 4 times that of myoglobin, the units would have a molecular weight very close to that of myoglobin and, therefore, would sediment at a rate similar to myoglobin.

**Acid Cleavage of Myoglobin**—The beef myoglobin was cleaved with HCl at an ionic strength of 0.04 (Fig. 4). Approximately 80 per cent of the hemin was cleaved between pH 4.5 and 4.0. The remaining iron porphyrin was removed by acidifying to pH 2.6. A definite break in the
HOMOGENEITY STUDIES

curve at pH 4.0 was obtained. The break at pH 4.0 is more easily seen if the expression log \((\text{concentration at pH 2}) - (\text{concentration at pH } x))/((\text{concentration at pH } x) - (\text{concentration at pH 7}))\) is plotted against pH (Fig. 4). The concentration at pH 2 and that at pH 7 are the concentrations of the hemin in acetone at complete and zero splitting, respectively. The concentration at pH \(x\) is the concentration of hemin at any point along the curve. From experiments with horse myoglobin and other hemoproteins (3) this indicates the presence of two components. Therefore, the results from the acid cleavage of beef myoglobin are in agreement with the electrophoretic homogeneity data.

The light absorption spectra of samples of the hemin cleaved at pH 4.3 and 2.5 were determined and both agreed with the spectrum of crystalline hemin. This indicates that the two components in the myoglobin preparation possess heme as a prosthetic group. The break in the curve can be interpreted to mean that the hemin of Substance A is more difficult to remove by acid cleavage and, therefore, the heme-protein linkages in this compound are different from those of myoglobin.

SUMMARY

Crystalline beef myoglobin isolated by ammonium sulfate fractionation was found to be electrophoretically inhomogeneous. The accompanying materials have been designated as Substances A and B. Substance A is present to an extent of 15 to 20 per cent, while Substance B amounts to less than 1 per cent of the protein mixture. Neither lead acetate fractionation nor repeated re-crystallization from ammonium sulfate removed Substances A and B.

Substance A possessed a light absorption spectrum similar to that of metmyoglobin and, when treated with pyridine and reducing agent, exhibited the reduced pyridine hemochromogen spectrum characteristic of hemin. This behavior is typical of a hemoprotein. Nothing is known of the chemical behavior of Substance B. Substances A and B were shown not to be hemoglobin.

Ultracentrifugal analysis of the myoglobin preparation indicated only one component. However, no conclusions could be drawn as to the molecular weight of Substances A and B, since mixtures of hemoglobin and myoglobin could not be resolved by ultracentrifugation.

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