Properties and Structural Consideration of Hemin $a^*$

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(Received for publication, December 3, 1959)

The mechanism by which the terminal respiratory enzyme, cytochrome oxidase, reduces molecular oxygen is still a matter of speculation. Fundamental to an understanding of this chemical mechanism is a clarification of the structure of the prosthetic group of this enzyme. Extensive work by many investigators (1–5) has clearly established that this prosthetic group is an iron porphyrin compound. This iron-containing tetrapyrrole has been labeled "hemin $a$" or "cytohemin." Attempts to establish the structure of hemin $a$ have been hampered by relatively poor isolation procedures which have resulted in material of doubtful purity. The studies of Falk and Rimington (6) have pointed out the great lability of the compounds involved.

Improved techniques for the isolation and purification of hemin $a$ (5, 7–9) and the development of paper chromatographic techniques (8) have made possible more definitive studies of the properties of hemin $a$. This paper will report studies on the infrared spectra and reactions of the functional groups of hemin $a$, providing new information on the structure of this compound.

METHODS

The preparation and purification of the hemin $a$ components were carried out as previously described (5, 7, 9). Further purification was done, when required, by means of rechromatography on silicic acid columns (9).

The hemins were characterized and their purities checked by paper chromatography (8, 9). The chromatographic systems employed were: toluene-acetic acid; picric acid-benzene-isopropyl alcohol; lutidine-water; and pyridine-ammonium hydroxyl-isopropyl alcohol.

The hemins were converted to their respective porphyrins by the method previously described (7). The HCl number was determined by extracting an ether solution of porphyrin with a similar pyridine solution of the tetrapyrrole.

Hemin $a$ or porphyrin $a$ was prepared by adding 2.0 mg of solid hydroxylamine hydrochloride and 4.0 mg of sodium acetate to a solution of approximately 25 $\mu$g of the compound in 3.0 ml of pyridine. The mixture was stirred and allowed to stand at room temperature for 5 minutes. Hemin $a$ or porphyrin $a$ was reduced by a few milligrams of solid sodium borohydride added to a similar pyridine solution of the tetrapyrrole.

Samples of chromatographically pure hemins were analyzed for iron according to a modification of the method described by Drabkin (11). The dried hemin sample was digested with 1 ml of $\text{H}_2\text{SO}_4$ and 3 drops of 30% $\text{H}_2\text{O}_2$ for about 5 minutes. Hydrogen peroxide was added time to time to complete decolorization. Boiling was continued for at least 10 minutes after the last peroxide addition. The solution was cooled and diluted with 5 ml of water. In order to adjust the pH to a value of 4.3, 6 ml of saturated sodium acetate were added. The mixture was transferred quantitatively to a 25-ml volumetric flask and 0.4 ml of 1% ascorbic acid and 2 ml of 0.1% $o$-phenanthroline were added in rapid succession. The volume was brought to the mark and after thorough mixing the solution was allowed to stand, while stoppered, for a minimum of 1½ hrs. The optical density was read at 540 nm against a reagent blank. These values were compared with iron standards.

The infrared spectra were recorded on a Perkin-Elmer model 21 infrared spectrophotometer. Samples containing 100 $\mu$g of hemin $a$ were dissolved in benzene-acetone and mixed with 25 mg of lyophilized potassium bromide. The slurry was then lyophilized and about 8 mg of the mixture pressed into a clear pellet (0.065 inch in diameter) which was used to obtain the spectrum.

RESULTS

The hemin prepared by column chromatography was checked for purity by the use of paper chromatography. The presence of small amounts of contaminating protohemin in the hemin $a$ preparation could readily be detected on the paper chromatograms even though it was not apparent on spectral analysis. With the aid of this technique, it was noted that a pure sample of hemin $a$, on standing in chloroform, was converted to a compound distinguishable from hemin $a$ in all solvents (see Table 1). The conversion product, hemin $a'$, had a higher $R_F$ value than hemin $a$ in all solvents as shown in Table 1.

* This research was supported by the National Heart Institute, United States Public Health Service, Grant No. 1322, and the Life Insurance Medical Research Fund.

† Supported by a Senior Research Fellowship SF 47 of the United States Public Health Service.
April 1960

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TABLE I

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Hemin a</th>
<th>Hemin a'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene-acetic acid</td>
<td>0.4</td>
<td>0.73</td>
</tr>
<tr>
<td>Benzene-isopropl alcohol</td>
<td>0.5</td>
<td>0.84</td>
</tr>
<tr>
<td>Lutidine-water</td>
<td>0.81</td>
<td>0.95</td>
</tr>
<tr>
<td>Pyridine-NH₂OH-isopropyl alcohol</td>
<td>0.73</td>
<td>0.92</td>
</tr>
</tbody>
</table>

In all instances ascending chromatography was employed as previously described (8, 9).

Chloroform solutions of pure hemin a, checked by paper chromatography, were dried in a stream of nitrogen and used for further studies. The molar extinction coefficients of the reduced and oxidized pyridine hemochromogens of hemin a, based on the iron content of the hemin samples used, are shown in Fig. 1. The reduced hemochromogen of hemin a has a single absorption maximum in the visible region at 587 μm and the molar extinction coefficient at this wavelength was determined to be 0.26 × 10⁺⁸ cm² per mole. In the Soret region of the spectrum, the maximum is at 430 μm and the extinction coefficient is 1.21 × 10⁺⁸ cm² per mole. To obtain the molecular weight of hemin a, samples of the pure hemin were weighed and the extinction of the hemochromogen determined. The molecular weight was found to be 880.

In Fig. 2 the infrared spectrum of hemin a in the region 5.0 to 7.0 μm is shown. The absorption in this region of the spectrum is attributed to the presence of carbonyl groups. The absorption at 5.86 μm is assigned to the carbonyl of the carboxyl group. Protoporphyrin, which has two carboxyl groups, has an absorption maximum at the same position, and a sample prepared in the same manner as the hemin a gave an equal absorbency on a molar basis.

The absorbancy at 6.00 μm is assigned to either aldehyde or ketone carbonyl groups. The intensity of the absorbancy at 5.86 μm and 6.00 μm for hemin a was very nearly the same.

The spectrum of the porphyrin prepared from pure hemin a is illustrated in Fig. 3. The relative intensities of the four absorption maxima of this porphyrin are III > IV > II > I, which classified porphyrin a as having an oxyrhodo type spectrum (15). The spectrum of the oxime is also shown in Fig. 3. The conversion of the formyl group of porphyrin a to an oxime causes a marked alteration of the spectrum, resulting in a rhodo type spectrum (III > IV > II > I). The positions of the absorption maxima are also altered.

When porphyrin a, dissolved in anhydrous pyridine, was treated with a few mg of solid sodium borohydride, the aldehyde and ketone groups of the porphyrin were reduced to the alcohols. This reduction also results in spectral changes. The spectrum of the reduced porphyrin is of the etio type (IV >
III > II > I) and the maxima have all shifted to shorter wave lengths as shown in Fig. 3.

Hydriodic acid will add to isolated double bonds or to vinyl side chains yielding a saturated compound. Porphyrin a treated with HI results in a porphyrin with a rhodo type spectrum, but when this product is converted to an oxime, a porphyrin with an alio type spectrum results. The position of the $\alpha$ absorption peak of hemin a and derivatives of hemin a are given in Table II.

The HCl number of porphyrin a was determined to be about 15, whereas porphyrin a' had an HCl number of about 5. It should be mentioned that cryptoporphyrin a (7) has a low HCl number of about 1.8.

### Table II

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wave length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemin a</td>
<td>587</td>
</tr>
<tr>
<td>Oxime</td>
<td>571</td>
</tr>
<tr>
<td>BrH reduction</td>
<td>582</td>
</tr>
<tr>
<td>Br$_2$ addition</td>
<td>582</td>
</tr>
<tr>
<td>Dimedon</td>
<td>558</td>
</tr>
<tr>
<td>Acetylation</td>
<td>582</td>
</tr>
<tr>
<td>Chlorocruorohemin</td>
<td>583*</td>
</tr>
<tr>
<td>Oxime</td>
<td>501*</td>
</tr>
</tbody>
</table>

* Lemberg and Falk (15).

**DISCUSSION**

Extensive studies in a number of laboratories have provided several pertinent facts concerning the structure of hemin a. The molecular weight of hemin a, which was determined to be 880, is a value which is in reasonable agreement with other studies (2, 12).

Hemin a is a dicarboxylic acid as indicated by paper chromatography (8) and the data presented in this paper. These two carboxyl groups are part of the propionic acid side chains (13, 14). The notable work of the Ottawa group (14) on the structure of the deuteroporphyrin derived from hemin a by resorcinol fusion, assigns the position and describes the nature of five of the eight groups substituted on porphyrin a. These are three methyl groups and two propionic acid side chains.

It remains then, to decide what three groups are substituted on the porphyrin nucleus and to determine their arrangement. From the spectrum of hemin a, it is apparent that more than one electron withdrawing group is substituted in the three positions whose substituents are unknown. The $\alpha$-peak of the pyridine hemochromogen is an excellent index of the number of about five of the eight groups substituted on porphyrin a. These are three methyl groups and two propionic acid side chains.

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The presence of an aldehyde group in resonance with the porphyrin nucleus of hemin a is demonstrated by the formation of the dimedon derivative of hemin a. This reaction of hemin a with methone is considered to be specific for aldehydes and thus demonstrates the presence of a formyl group. Other studies employing less specific reactions have also indicated the presence of this group (3, 15).

Treatment with hydrogen iodide causes a marked shift in the spectrum of porphyrin a. Bromine will add to hemin a and the compound which results has a hemochromogen spectrum whose $\alpha$-peak is shifted to a lower wave length. This shift of 5 m$\mu$ is what would be expected on loss of a double bond.

A comparison of the $\alpha$-peak of the pyridine hemochromogen of hemin a and chlorocruorohemin show a difference of 4 m$\mu$ whereas their respective oximes have an even greater difference of about 10 m$\mu$. These differences suggest that a formyl group and vinyl group, the two electron withdrawing groups present in the structure of chlorocruorohemin, are not the only electron withdrawing groups in the hemin a molecule.

Hemin a can be acetylated and a product is obtained with altered chromatographic mobility. This acetylation also results in a shift of the hemochromogen $\alpha$-peak to shorter wave lengths. This shift of the $\alpha$-peak indicates that an electron withdrawing group has been removed from resonance with the porphyrin nucleus.

Borohydride reduction, which specifically reduces ketones and aldehydes, on reaction with hemin a, gives a compound whose $\alpha$-peak is at 552 m$\mu$. The position of this peak is consistent with a compound which has but a single double bond remaining in resonance on the side chains. The third electron withdrawing group then appears to be removed by reduction with sodium borohydride. The acetylation of hemin a appears to have a greater effect on the spectrum than would be predicted for the acetylation of a simple alcoholic group. A comparison of the infrared spectrum of hemin a and other compounds suggests that the absorbancy at 6.00 m$\mu$ may be due to both a ketone and an aldehyde group.

The third electron withdrawing group present in hemin a may be $\text{C}_2\text{H}_4\text{O}_2$. Such a group would give results compatible with all the data. This group could account for the $\beta$ and $\alpha$ porphyrins observed by Lemberg and Stewart (16) as well as our hemin a' fraction, since it could readily enolize and be converted to an isomeric form. Since the alcohol group makes an asymmetric carbon atom, one would expect hemin a to be optically active. We could not detect any optical activity in hemin a. This could also be due to the fact that enolization gives rise to a racemic mixture. It must be emphasized, however, that optical rotation measurements of intensely colored compounds, such as hemin a, are difficult.

The spectrum of the porphyrin a and its derivatives can give us information concerning the arrangement of these groups on the porphyrin nucleus (15). Treatment of porphyrin a with hydriodic acid converts the spectrum of the porphyrin from an oxyrhodo to rhodo type spectrum. This indicates that the double bond has a rhodofying effect and must be on the pyrrole opposite to that containing the formyl group. Thus, the formyl and vinyl groups must occupy positions 4 and 8 of the pyrrole ring.
The R and R' groups in the proposed structure together contain 13 carbon atoms. The available evidence does not enable us to determine the distribution of the carbon atoms in the two alkane groups.

**SUMMARY**

It has been shown that pure hemin a, on standing in chloroform, is converted to a compound with spectral properties identical to hemin a, but with altered chromatographic properties.

The extinction coefficient of the reduced pyridine hemochromogen of hemin a was determined to be $0.26 \times 10^4$ cm$^2$ per mole at 587 nm and $1.21 \times 10^4$ cm$^2$ per mole at 430 nm. The molecular weight of the hemin a chloride was estimated to be 880.

A number of derivatives of hemin a were made, including the bromine addition product, borohydride reduction product, dimedon, oxime, and acetylated derivatives.

The results were discussed and a possible structure for hemin a was proposed.

Acknowledgment—The authors wish to express their appreciation to Mr. Arthur Behringer and Dr. William Mason for assistance in obtaining the infrared spectra.

**REFERENCES**

Properties and Structural Consideration of Hemin a
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