THE EXTRACTION AND PAPER CHROMATOGRAPHY
OF HEMINS*

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The classical procedure for the identification of hemins as prosthetic
groups is to isolate the hemins, convert them into porphyrin esters, and
identify the esters by their spectra and melting points. This procedure
usually requires considerable amounts of material, gives poor yields,
and the melting points have not always been definitive (1). Chromato-
graphic methods have aided in establishing the homogeneity of porphyrins
and porphyrin esters (2-5). Nevertheless, the multiple porphyrins which
may result from the conditions required to convert hemins to porphyrins
(1, 6, 7) and the instability of some porphyrins make this general procedure
for hemin identification less certain than if the hemins themselves could be
identified.

More recently, methods of column chromatography have been developed
which can separate protohemin from hemin a (8) and even from two hemin
a types (9). These techniques were essentially preparative, but a micro-
procedure for the identification of hemin prosthetic groups prepared from
small amounts of hemoproteins would seem useful. Chu and Chu (10)
have reported solvent systems for the separation of hemins by paper
chromatography, but the hemins used were pure samples which had been
prepared by large scale purification procedures.

The method reported here deals with hemins obtained directly from
small quantities of cytochrome c (11), cytochrome oxidase preparations
(12), hemoglobin, catalase (13), and various rat tissues. Special procedures
for the separation of the hemins from their hemoproteins are reported,
and new acid solvent systems for the paper chromatography of the hemins
are described. The separation of various hemins from the above prepara-
tions and tissues is demonstrated.

EXPERIMENTAL

Separation of Hemins from Lipide-Free Material

The hemin was separated from catalase and crystalline normal adult
hemoglobin by cleavage with acidified acetone (9). The acetone solution

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of hemin was diluted with an equal volume of ethyl ether, and then washed with an equal volume of 5 per cent NaCl and concentrated or diluted as necessary for chromatography. Only freshly prepared solutions of hemin were used.

A modification of Paul's procedure (14) for the separation of the hemin from cytochrome c was employed. To 10 ml. of a cytochrome c solution \(2 \times 10^{-4}\) M, 2 ml. of glacial acetic acid and solid silver nitrate sufficient to saturate the solution were added. The flask was shaken vigorously for 5 minutes and allowed to stand in the dark at room temperature for 15 hours. The solution was decanted from the excess solid silver nitrate and then extracted with an equal volume of ether containing 20 per cent (by volume) of acetic acid in a separatory funnel. The ether phase was separated and the extraction procedure repeated four times. The combined ether extracts were then washed with an equal volume of 5 per cent sodium acetate solution. The ether solution of the prosthetic group of cytochrome c, appropriately concentrated or diluted, was used directly for chromatography.

**Separation of Hemins from Lipide-Containing Material**

**Oxidase Preparation**—The cytochrome oxidase preparation was treated as described previously (9), but no steps beyond the stage of washed ether solution were needed. This ether solution may be concentrated or diluted as necessary for paper chromatography.

**Tissues**—In the case of rat tissues, it was first essential to remove excess hemoglobin and myoglobin, since the large quantities of protohemin derived from these compounds obscured the smaller amounts of other hemins during chromatography. For this purpose a 4 per cent homogenate of the tissue was prepared with a solution of 20 per cent sucrose containing 0.25 per cent saponin. The mixture was centrifuged and the residue washed three times with 10 volumes of the sucrose-saponin solution. The preparation was then ready for removal of excess lipides. Toward this end the above residue was first partially dehydrated by the addition of 30 volumes of acetone and was then extracted twice with the same volume of chloroform-methanol (2:1, v/v). The residue was finally washed once with 30 volumes of acetone.

After the addition of 1 ml. of water per gm. of acetone powder, the hemins were extracted with 10 ml. of acidified acetone with stirring for 15 minutes. This operation was repeated until the extract was no longer colored. The combined acetone extracts were then diluted with an equal volume of ether in a separatory funnel and washed three times with equal volumes of 5 per cent NaCl. The ether solution was concentrated or diluted as necessary for paper chromatography.
Chromatography—Four different solvent systems were employed for the separation of the hemins by paper chromatography. Two solvent systems partitioned the hemins between a stationary acid aqueous phase and a mobile non-polar phase. The first of the acid solvents was composed of 2 ml. of 99 per cent formic acid and 10 ml. of hexane, made up to 100 ml. with chloroform. The second acid solvent consisted of benzene saturated with picric acid and containing 4 per cent isopropyl alcohol (v/v).

One of the basic solvents consisted of 55 ml. of 2,6-lutidine and 45 ml. of water; the other consisted of pyridine, isopropyl alcohol, and 8 N ammonium hydroxide in the ratio of 1:2:2 by volume.

Whatman No. 1 paper was used with all solvents. In the case of the pyridine-isopropyl alcohol-NH₄OH solvent, the paper was first impregnated with silicone grease. This was accomplished by passing the paper through a petroleum ether solution saturated with Dow-Corning silicone grease and allowing the petroleum ether to evaporate from the paper before use.

The chromatograms for which the non-polar acid solvents were employed were run in glass chromatography jars lined with paper immersed in the solvent. In these systems the descending technique of development was used. The hemins were spotted with micropipettes on paper 7.5 cm. in width, and the solvent was usually allowed to run approximately 30 cm. For greater separation of the hemins the solvent may be allowed to drip off the end of the paper. With the basic solvents, paper-lined museum jars were used, and the solvent was allowed to ascend the paper to a height of 15 to 18 cm. from the point at which the hemins were applied.

The benzidine reaction was used to locate the hemin spots. The reagent was prepared by shaking an excess of solid benzidine hydrochloride with absolute methanol for 1 minute and allowing the benzidine to settle. To 25 ml. of supernatant solution were added 12.5 ml. of distilled water, 5 ml. of 3 per cent hydrogen peroxide, and 2.5 ml. of glacial acetic acid. The freshly prepared reagent was sprayed lightly on the dry papers, and the development of the blue spots was rapid. Since the paper and spots discolor with time, it is desirable to outline the spots shortly after development. The reagent has remarkable sensitivity. A spot containing 0.006 \( \gamma \) of hemin spread over an area of 1.5 cm. in diameter can be detected, while 0.05 to 0.1 \( \gamma \) gives a very intense spot and was the concentration of hemin used in most of the studies.

Results

Both the acidic and basic solvent systems distinguish protohemin from the \( \alpha \) type hemins, and the non-polar acid solvents distinguish protohemin from the hematohemins derived from cytochrome \( c \). The formic acid-containing system (Fig. 1) can effect the separation of hemins \( a_1 \) and \( a_2 \), as
well as the hematohemins $c_1$ and $c_2$, from cytochrome $c$. Column 2, Fig. 1, shows the marked effect caused by the presence of contaminating lipides on the chromatographic behavior of the hemins. Although the preparation of hemins from cytochrome oxidase, whose chromatography is illustrated in Fig. 1, still contains some polar lipides, it is obviously sufficiently free from interfering lipides to permit satisfactory separation of the hemins.

**Fig. 1.** Diagrammatic representation of hemin chromatography with the chloroform-hexane-formic acid solvent system. Column 1, hemins of cytochrome oxidase preparation with excess lipides removed; Column 2, same as Column 1 but without removal of the lipides; Column 3, hemins from purified cytochrome $c$ preparation; Column 4, same as Column 1 but the solvent was allowed to run off paper. Symbols, $P =$ protohemin, $A_1 =$ hemin $a_1$, $A_2 =$ hemin $a_2$, $C_1 =$ hematohemin $c_1$, and $C_2 =$ hematohemin $c_2$.

There are certain disadvantages of the formic acid-containing solvent in contrast to the picric acid solvent. The former is more sensitive to temperature changes, is less stable, and separates into two phases on standing. The picric acid solvent leaves the paper stained yellow, and, upon being sprayed with the benzidine reagent, the spots appear as brown areas on the

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1 The solvent systems employed in these studies were also applied to the porphyrins. In the formic acid solvent, the free porphyrins did not move, and the methyl esters moved with very high $R_f$ values. Uroporphyrin, the slowest moving of the porphyrins, had an $R_f$ value of 0.82. The methyl esters of proto-, copro-, and uroporphyrins were more successfully separated in benzene saturated with picric acid.
yellow background. This makes a permanent record, since the spots do not fade. The paper does not discolor, which is in contrast to the fading of spots and discoloration of the paper which occur with the other solvent systems.

Fig. 2 illustrates the movements and separations of the various hemins in basic solvent systems. Of special interest is the comparison of the mobility of the hemins $a$ (Columns 6 and 7) with protohemin and its mono- and diesters (Columns 1, 2, and 3). It is indicated that the hemins $a$ are weak acids moving in the system in the same manner as a monocarboxylic acid hemin. It is to be noted that the basic solvent systems do not effect a separation of the two hemins $a$, but that the pyridine $\text{NH}_4\text{OH}$-isopropyl alcohol system produces a separation of the hematohemins $c_1$ and $c_2$. In the basic as well as the acid systems, the presence of excess lipides (Column 5) prevents separation of the hemins.

The chromatographic methods may be applied in a semiquantitative way to the hemins, which may be derived from a variety of tissues. Before liberation of the hemins by acidified acetone, it is necessary to remove excess lipides by the method described. The hemins obtained from six different tissues of the rat were chromatographed in the benzene-isopropyl
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alcohol-picric acid system. Maximal separation of protohemin from the hemins a was obtained when the concentration of isopropyl alcohol was 4 per cent.

Protohemin was the largest component present (Table I) in all the tissues investigated. These tissues also contained hemins which moved in the different solvent systems with \( R_F \) values identical with that of the hemins a. Finally, smaller amounts of a hemin with a very high \( R_F \) value could be detected in most of the tissues. This hemin appears to be the same as that previously isolated from heart muscle and is believed to be a protohemin ester of an unidentified lipide material (15).

**Table I**

_Hemins Present in Acidified Acetone Extracts of Some Rat Tissues_

The tissues were freed from excess hemoglobin and lipide before extraction with acidified acetone, as described in the text. The benzene-isopropyl alcohol-picric acid solvent system was used for chromatography.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Protohemin, ( R_F = 0.5 )</th>
<th>Hemins a, ( R_F = 0.2 )</th>
<th>Protohemin ester, ( R_F = 0.92 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>++++*</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Liver</td>
<td>+++</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Lung</td>
<td>+++</td>
<td>±</td>
<td>−</td>
</tr>
<tr>
<td>Intestine</td>
<td>++++</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Heart muscle</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Spleen</td>
<td>++++</td>
<td>+</td>
<td>±</td>
</tr>
</tbody>
</table>

* Symbols: −, not detectable; ±, detectable; + to ++++, increasing concentrations present.

**DISCUSSION**

According to the proposed structures of porphyrin a, this compound is a dicarboxylic acid containing a formyl group and probably a long chain aliphatic group (16, 17). It is interesting to note that, in the basic solvents in which the porphyrins move according to the number of carboxyl groups contained in the compound, the hemins a move as monocarboxylic acids. Conversely, in the non-polar acid solvent systems these compounds move with low \( R_F \) values, even though the presence of the aliphatic group should make them more soluble in the mobile non-polar solvent. This suggests that the structure of hemin a varies from protohemin not only in the additional formyl group and aliphatic chain but in the presence of another polar functional group.

In a study of the porphyrins obtained from hemins extracted from ox kidney and liver as well as cow udder, Lemberg _et al._ (18) found that the porphyrin a derived from these tissues differed in its absorption spectrum.
from that derived from heart muscle. Our results with rat tissues indicate that in two different solvent systems the hemins from the various tissues appear to have identical $R_F$ values. In the visible region, these hemins also have absorption maxima in the same positions. Of the suggestions made by these authors, our results would support the belief that the apparent differences noted in the porphyrins were due to variations in tissue composition and the effects of impurities on the properties of the products observed.

The same authors also found evidence for the presence of a protoporphyrin ester in kidney. We have been able to isolate a protohemin ester from heart muscle (15), and this compound can be shown to be present in almost all the tissues of the rat, as noted in Table I.

The multiple hemins obtained from a purified preparation of cytochrome $c$ have been observed earlier (19, 20). The failure of Chu and Chu (10) to observe separation in their systems may be referable to the small yield of hemin obtained by the procedure employed in the cleavage of the hemin from the protein, or to the method of detection of the hemin on the paper.

SUMMARY

Methods are outlined for the cleavage and extraction of the prosthetic groups from small amounts of hemoprotein preparations. These methods for the extraction of the hemins make it possible to separate the hemins by paper chromatography.

New acid solvent systems for the paper chromatography of the hemins are described.

From a purified cytochrome $c$ preparation two hemins could be identified by paper chromatography. The two hemins $a$ previously reported (9) were also separable by these techniques. The hemin derived from catalase moved with $R_F$ values identical with those of the hemin derived from hemoglobin.

The separation of hemins from various rat tissues is demonstrated.

BIBLIOGRAPHY

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