PARTITION CHROMATOGRAPHY OF HEMINS. SEPARATION OF THE PROSTHETIC GROUPS OF CYTOCHROMES a AND a₃*

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The earlier work of Keilin and Hartree (1) and the later reports of Ball and coworkers (2) and of Chance (3–5) leave little doubt that cytochromes a and a₃ are two different hemoproteins. Comparison of the effect of carbon monoxide, as observed spectroscopically at 605 µm and in the Soret region, indicates pronounced differences in the extinctions of these components and suggests either different hemin prosthetic groups or very different types of linkages between hemin and protein in cytochromes a and a₃.

Other workers were concerned with the dichroic ("green") hemin of heart muscle. Thus Negelein (6) separated a hemin which as a reduced pyridine hemochromogen possessed an absorption band at 587 µm. Roche and Benevent (7) questioned whether this hemin was derived unchanged from the tissue, and the cryptoporphyrin prepared from the hemin was considered by Negelein (8, 9) to be an artifact. More recent work (10–12) leaves no doubt that such a "green" hemin can be derived from tissue and that it is characterized by a reduced pyridine hemochromogen having absorption peaks at 430 and 587 µm. In addition, Dannenberg and Kiese (13) have isolated a hemin from a partially purified cytochrome oxidase with these absorption characteristics, and Person and coworkers (14) found a similar spectrum upon addition of pyridine to an oxidase preparation. The work cited implies that there is a single prosthetic group (hemin a) for cytochromes a and a₃.

Falk and Rimington (11), in a thorough investigation of the porphyrins derived from hemin a, demonstrated that "porphyrin a" was labile to acid and is probably a mixture of at least four porphyrins. Thus porphyrin separation could not lead to valid conclusions as to the possible multiplicity of hemin prosthetic groups, and these investigators were unsuccessful in their attempts to purify hemin a by column chromatography. Most recently Kiese and Kurz (15) have described a chromatographic method

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which has separated hemin \( a \) from protohemin. Only a single hemin \( a \) fraction was obtained by this method.

It is apparent that a method for separating hemins rather than the porphyrins might be fruitful in supplying information concerning the prosthetic groups of cytochromes \( a \) and \( a_3 \). The object of this paper is to describe a procedure for the partition chromatography of hemins and to demonstrate by this method that cytochromes \( a \) and \( a_3 \) have closely related but different prosthetic groups.

**EXPERIMENTAL**

**Column Chromatography**

*Preparation of Adsorbent*—Silicic acid was the solid adsorbent used to hold the stationary aqueous phase. 500 gm. of reagent grade silicic acid (Merck or Mallinckrodt) are thoroughly mixed with 800 ml. of acid ethanol (400 ml. of c.p. hydrochloric acid diluted to 2400 ml. with 95 per cent ethanol). The mixture is filtered through a Büchner funnel, and the washing procedure is repeated at least three times. The filtrate from the final washing should be colorless. The silicic acid is then washed three times in the same manner with 700 ml. portions of 95 per cent ethanol, and finally three times with 800 ml. portions of diethyl ether. It is then spread on clean white paper and allowed to dry for 24 to 48 hours. The material is then passed through an 80 mesh sieve and dried in a vacuum desiccator.

*Chromatography*—To 50 gm. of the silicic acid prepared in this way, 20 ml. of 0.15 N hydrochloric acid are added, and the mixture is stirred to a homogeneous powder. To this powder 80 ml. of reagent grade chloroform are added, and the mixture is stirred until a fine slurry is obtained. This slurry is poured into a chromatographic tube 3.5 cm. in diameter and the silicic acid is then washed three times in the same manner with 700 ml. portions of 95 per cent ethanol, and finally three times with 800 ml. portions of diethyl ether. It is then spread on clean white paper and allowed to dry for 24 to 48 hours. The material is then passed through an 80 mesh sieve and dried in a vacuum desiccator.

The following procedure is described for the chromatography of hemin solutions which are contaminated with lipides as the result of the tissue extraction procedure. To the column wet with chloroform, 10 ml. of \( n \)-hexane are added. When 2 to 5 ml. of hexane remain on top of the column, an equivalent volume of a concentrated chloroform solution of the hemins is delivered carefully onto the column (most of the chloroform settles below the hexane layer and precipitation of the hemins is avoided). As the last of the liquid disappears into the column, about 10 ml. of a 1:1 (volume per volume) chloroform-hexane mixture are used to rinse the walls of the tube above the adsorbent. 100 ml. of the chloroform-hexane mixture are then poured onto the column, and, as the last of this solvent passes into the adsorbent, pure chloroform is used to elute the hemins. The use
of hexane accomplishes the removal of lipides from the column prior to the elution of the hemins by pure chloroform.

The steps involving hexane and hexane-chloroform are omitted in the chromatography of lipide-free hemin solutions.

**Extraction of Hemins**—The starting material for the investigation of the prosthetic groups of cytochromes a and a₃ was the purified cytochrome oxidase preparation of Smith and Stotz (16). The precipitate collected after dilution of the Type I oxidase preparation (16) was extracted three times with 10 volumes of acetone, then three times with the same volume of chloroform-methanol (2:1 volume per volume), and once again with acetone. These extractions served to remove water and excess lipides. For each gm. of dry residue, 100 ml. of acid acetone (1.8 ml. of concentrated hydrochloric acid per 100 ml. of acetone) are used to extract the hemins. The solid was extracted with stirring at 4°C for 15 minutes; this was repeated with fresh solvent until the extract was no longer colored. The combined acid acetone extracts were then diluted with an equal volume of peroxide-free ether in a separatory funnel, and the solution was washed with 10 volumes of 5 per cent sodium chloride solution. The washing procedure was repeated four times. The washed ether solution of hemins was evaporated to an amorphous solid under vacuum. This was
dissolved in a minimal amount of boiling chloroform and filtered. The clear concentrated chloroform solution of hemins was then chromatographed as described previously.

**Results**

The hemins, being highly colored, separate into easily visible bands on the column; they may be collected as they are eluted from the column.

![Graph showing the reduced pyridine hemochromogen spectra of the hemins prepared from cytochrome oxidase.](http://www.jbc.org/)

\[ \text{Fig. 2. The reduced pyridine hemochromogen spectra of the hemins prepared from cytochrome oxidase.} \]

\[ \text{○, represents Fraction 1 from the column; \times, represents Fractions 2 and 3. The cuvettes contained the hemin dissolved in 2 ml. of 0.05 NaOH and 1 ml. of pyridine. The hemochromogen solutions employed for measurements in the visible region were diluted 1:3 for Fraction 1 and 1:5 for Fractions 2 and 3 with alkali and pyridine for measurements in the Soret region.} \]

The eluent may also be collected with an automatic fraction collector and the amount of hemin in successive tubes measured with the Beckman spectrophotometer. All the hemins encountered in this work were found to follow Beer's law of dilution in chloroform at 410 μm, which was the point of maximal absorption for the α type hemins.

Fig. 1 shows a typical chromatograph of the hemins extracted from a cytochrome oxidase preparation. Three hemins, labeled Fractions 1, 2, and 3, are evident. The spectra of the reduced pyridine hemochromogens
of these three hemins appear in Fig. 2. The hemochromogen from Fraction 1 has absorption peaks at 557, 527, and 420 m\(\mu\), which are characteristic for pyridine protohemochromogen, and the protohemin undoubtedly arose from the hemoprotein known to be present in the oxidase preparation which absorbs in the region of 560 m\(\mu\) (16). The hemochromogens of Fractions 2 and 3, however, with absorption peaks at 587 and 430 m\(\mu\), are similar to those commonly considered to be derived from hemin \(a\). Fractions 2 and 3 are therefore considered to be derived from cytochromes \(a\) and \(a_3\).

To test the possibility that one of the hemin \(a\) components gave rise to the other as an artifact of the treatment to which they were subjected, each of the \(a\) hemins taken from the column was again carried through the entire procedure, including the acid acetone treatment. However, each hemin yielded a single hemin on retreatment, and the product in each case had the same chromatographic and spectral properties as the starting material. Thus these two hemins are not interconvertible during the isolation procedure, which supports the conclusion that they arose from separate hemoproteins.

**DISCUSSION**

The finding that two hemins of the \(a\) type may be derived from a cytochrome oxidase preparation leads to the conclusion that they represent the prosthetic groups of the two hemoproteins, cytochromes \(a\) and \(a_3\). In view of the similar spectral properties of the two \(a\) type hemins, the different extinction coefficients of cytochromes \(a\) and \(a_3\) (1, 3–5) and the difference in their abilities to combine with inhibitors must be due more to differences in the linkages between hemin and protein than to the differences in the tetrapyrrole structures. This is supported by the finding that the rates of liberation of the \(a\) hemins from the hemoproteins of the cytochrome oxidase by acid acetone are very different (17).

The present study supplies no direct evidence as to which hemin \(a\) is derived from cytochrome \(a_3\). In this connection it is of interest that the areas under the curves in the chromatograph of Fig. 1 indicate approximately equal amounts of the two \(a\) hemins, and that Chance (5) has estimated that there are essentially equimolar amounts of cytochromes \(a\) and \(a_3\) in a heart muscle preparation. This comparison seems valid, since it has been shown (16) that the proportions of cytochromes \(a\) and \(a_3\) are the same in heart muscle particles and in the purified oxidase preparation.

1 The reduced pyridine hemochromogen of the hemin \(a\) fraction prepared by Kiese and Kurz (15) possesses minor absorption peaks at 534 and 508 m\(\mu\) as well as the major ones at 587 and 430 m\(\mu\) noted by others. These minor peaks are not evident in either of the \(a\) hemins described in this paper.
The close spectral similarity of the two \( \alpha \) hemins further indicates that their differences may be related more to the side chains of the hemin nucleus, which are not part of the resonating porphyrin structure, than to essential changes in the porphyrin nucleus itself.

**SUMMARY**

1. A method for the partition chromatography of hemins is described.
2. Application of the method to the hemins liberated from a purified cytochrome oxidase preparation yielded two hemins of the \( \alpha \) type. It was concluded that these hemins represent the prosthetic groups of cytochromes \( \alpha \) and \( \alpha_3 \).
3. The relationship of the findings to the difference in properties of cytochromes \( \alpha \) and \( \alpha_3 \) are discussed.

**BIBLIOGRAPHY**

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