Isolation and Characterization of the Unique Prosthetic Group of a Green Hemoprotein from Human Erythrocytes*

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A simplified and streamlined purification scheme has been developed for the large scale isolation of a green hemoprotein from human erythrocytes. The isolation procedure involves hypotonic lysis, freezing of the hemolysate at -60°, centrifugation, direct chromatography of the supernatant on DEAE-cellulose, and subsequent cation exchange, anion exchange, and gel filtration chromatography. Approximately 2 pmol of this anionic hemoprotein were isolated per liter of packed erythrocytes.

The previous inability to separate the prosthetic group from the protein by conventional solvent extraction procedures was shown to be a consequence of the highly polar character of the heme and not due to covalent linkage between heme and protein. The polar nature and marked lability of the heme necessitated development of techniques for the extraction, purification, and derivatization of the prosthetic group. The heme was separated from the protein by membrane filtration in the presence of pyridine and alkali or by disc gel electrophoresis in the presence of cyanide. The heme was methylated with trimethyl-tetrafluoroborate and further derivatized. The heme derivatives were purified on columns of Sephadex LH-20 or alumina.

Chromatography of the heme, heme methyl ester, acetylated heme ester, and the corresponding porphyrin derivatives suggests that the heme contains three carboxyl groups and one or more polar, acetylatable functional groups, probably hydroxyl groups. Spectral characterization of these compounds, as well as the derivatives resulting from reaction of the heme with NH₂OH, NaHSO₃, and Na₂S₂O₃, show that the prosthetic group is a previously undescribed, formyl-containing heme that can be clearly distinguished from heme α, Spirographis heme, and all other naturally occurring prosthetic groups.

A green hemoprotein with spectral properties distinct from any previously known protein was isolated from human erythrocytes by Morrison in 1961 (1). The protein was obtained in soluble form by freezing and thawing red cells that had been suspended in water containing a little toluene. In collaboration with Dr. Morrison, we further purified and studied this protein (2). The molecular weight of the protein was shown to be approximately 21,000. No enzymatic activity was detected. The prosthetic group of this protein appeared to be a unique heme in that the spectrum of the pyridine hemochrome of the intact protein differed from that of all known naturally occurring hemes.

The study of the prosthetic group of this hemoprotein has been difficult because only microgram quantities of heme are present in the milligram quantities of protein thus far isolated, and the use of cation exchangers in the isolation procedure to remove the massive amounts of hemoglobin has limited the size of the preparation. Moreover, conventional solvent extraction procedures failed to liberate the heme from the protein, and the side chains of the heme appeared to be highly labile. In this paper we report greatly improved yields of hemoprotein by a simplified procedure suitable for very large scale isolations. We also report the extraction of heme from the protein, the purification of the heme, and the properties of this previously unreported, highly atypical prosthetic group. Preliminary reports of some phases of this work have previously appeared in abstract form (3-6).

RESULTS AND DISCUSSION

New Methods for Hemoprotein Isolation, Heme Isolation, and Heme Derivatization—The development of a number of new methods was necessary before it was possible to study the nature of the prosthetic group of this hemoprotein. Prerequisite

1 The experimental procedures are presented as a miniprint supplement immediately following this paper. For the convenience of those who prefer to obtain the methods in the form of 5 pages of full size photocopies, they are available as JBC Document No. 75M-1417. Orders should specify the title, authors, and reference to this paper, the JBC Document Number, and the number of copies desired. Orders should be addressed to The Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $1.00 per set of photocopies.

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to all studies was the development of a suitable large scale isolation procedure for the protein. The procedure described here is greatly improved over that described previously (2) in that larger preparations can be carried out more rapidly at less cost with a greater and more consistent yield. The greatly improved yield was in part a consequence of the substitution of DEAE-cellulose chromatography for the time-consuming cation exchange chromatography as the first step in the procedure and substitution of ultralfiltration for the ammonium sulfate precipitation and dialysis procedures. The use of a pH gradient in place of an ionic strength gradient to elute the hemoprotein from DEAE-cellulose greatly reduced the amount of contaminating salt which had to be subsequently removed from the sample and thus the time necessary to accomplish this desalting. However, the major factor responsible for allowing us to consistently obtain a good yield of hemoprotein was the use of the -60° treatment. We have demonstrated that freezing at -20° or omission of the freezing step resulted in no recovery of the green hemoprotein by the described procedure. The previously observed inconsistency of yield following rapid lysis in a dry ice-acetone bath (2) undoubtedly resulted from variation in the final temperature of the hemolsate.

The lability and the highly unusual properties of the heme prosthetic group of this protein necessitated the application of new techniques for its isolation, purification, and derivatization. The classical extraction of heme prosthetic groups into nonpolar solvent in the presence of acid was unsuitable for this protein because of the heme's acid lability and insolubility in nonpolar solvents. Our isolation of heme was accomplished by dissociating the heme from the protein with alkali and then separating heme from protein by membrane filtration, or by dissociating with cyanide and separating by disc gel electrophoresis. Likewise, the classical methods for heme purification were unsuitable for this water-soluble heme, and it was necessary to use anion exchange chromatography for purification.

Derivatization of the acid labile and polar heme to produce which could be highly purified and characterized by mass spectroscopy was not possible by methods in the literature. The only published methods for heme esterification use nonpolar solvent and mineral acids, conditions which were shown to alter the structure of the prosthetic group described in this paper; diazomethane has been reported to be completely unsuitable for heme methylation (16). Whereas methylation of porphyrins with diazomethane proceeds under mild conditions and without problem, a good preparation of porphyrin from the heme of the green hemoprotein was not possible because the acidic conditions for the conversion result in heme degradation. Thus, the present study of the prosthetic group was made possible by the development of the method for heme methylation in neutral, aqueous solution with trimethylxonium tetrafluoroborate (5, 15). The methylation appeared to proceed to near completion, as evidenced by the chloroform solubility of the heme compounds after the reaction and by the paper chromatographic analysis of the single identifiable product (Table IV) which showed the presence of no free carboxyl groups.

Our finding that the pyridine hemochromes of the isolated heme and heme methyl ester are spectrally indistinguishable from the pyridine hemochrome of the hemoprotein suggests that the structures employed for heme isolation, purification, and methylation did not alter the structure of the chromophore. We present the techniques described in this paper as general methods for the study of acid-labile or water-soluble heme prosthetic groups.

Noncovalent Nature of Heme to Protein Linkage—The inability of solvent extraction techniques to extract the heme results not from a covalent linkage between the protein and the prosthetic group but from the insolubility of the heme in ether and chloroform. The heme was found to be dissociated from the protein at pH 9.5 in the presence of 0.3 M cyanide (electrophoretic separation method), at pH 12 in the presence of pyridine (membrane filtration method), and in acidic conditions using ethyl acetate/acetic acid (extraction method). Cleavage under such diverse conditions would not be the expected behavior of amide, ester, thiol ester, ether, thioether, acetal, hemiacetal, N-acetal, disulfide, or any other linkage found in conjugated proteins.

Identification of Heme Carboxyl Groups—The heme isolated from the green hemoprotein by membrane filtration or by ethyl acetate/acetic acid extraction migrated somewhat slower in the lutidine/water/ammonia paper chromatographic system than did the dicarboxylic acid hemes: protoheme, heme a, and 2-formyl,4-vinyldeuteroheme. In this chromatographic system the migration of hemes is inversely related to the number of free carboxylic acid groups and is affected little by the presence of formyl or aliphatic hydroxyl groups. The Rf value of the prosthetic group is approximately 88% of the Rf values of the dicarboxylic acid hemes, which indicates that the polarity of the prosthetic group is comparable to nearly three carboxyl groups. After incubation with acetic acid (as described above), the modified heme migrated with the same Rf as protoheme. In analogy with heme a and protoheme, reaction of the prosthetic group with trimethylxonium tetrafluoroborate or with methanol/sulfuric acid yielded a product that migrated at the solvent front in the lutidine/water/ammonia system and migrated away from the origin in the propanol/kerosene system (Table IV). Such chromatographic behavior shows that the ion groups on the molecule are completely methylated by these procedures. Since the trimethylxonium tetrafluoroborate reaction in aqueous solution is specific for carboxyl groups (14), and since carboxyl groups are the only ionic residues which have been found on heme prosthetic groups, the chromatographic data are best explained by the presence of three carboxyl groups.

Presence of Formyl Group in Conjugation with Tetrapyrrole Ring—The hemoprotein, the isolated heme, and the heme methyl ester gave similar pyridine hemochrome spectra with absorbance maxima at wavelengths longer than those of monoformyldeuteroheme, but shorter than those of diformyldeuteroheme (Table I). Similarly, the absorbance maxima in chloroform solution of the porphyrin derived from the heme prosthetic group are intermediate between those of the monofomyldeuteroporphyrin and diformyldeuteroporphyrins (Table II). Reaction of the isolated heme with hydroxylamine at room temperature yields a derivative for which the pyridine hemochrome is found at 560 nm. This 19 nm shift toward shorter wavelengths corresponds to the magnitude of the shift for the oxime formation of a formyl group (Table III). The 30 nm shift in the α-peak of the pyridine hemochrome resulting from bisulfite addition and the 26 nm shift from dithionite reduction (Table I) likewise support the postulate that a formyl group is in conjugation with the tetrapyrrole nucleus. Only a formyl group would be expected to give the observed shifts. The shifts resulting from oxime formation of ketonic side chains are very
...soluble in chloroform. As described above, the chromato-
er ester of the prosthetic group have greater polarity than one
heme (Table IV), indicating that the side chains on the methyl
of protoheme, heme a, and 2-hydroxymethyl,4-vinyldeutero-
ph in nonpolar solvents at a slower rate than the methyl esters
methylation with oxonium salt, methanol/sulfuric acid, and
graphic properties show that the methylated product contains
no free carboxyl group. However, the products resulting from
methylation with hydrochloric acid reactions result in the modification
or cleavage of a polar portion of the molecule.

Acetic anhydride converts the methyl ester obtained by the
oxonium salt reaction from a polar compound with an \( R_f \) of 0.05 in the propanol/kerosene system to a derivative with an
\( R_f \) of 0.72, demonstrating that one or more polar groups of the
prosthetic group are acetylatable and suggesting the presence
the derivative is still a more polar compound than 2,4-diformyl-
porphyrin as evidenced by its failure to migrate in the
chloroform/kerosene solvent. It is unclear whether this polar,
acetylatable group is the same or a different group than the
second electron-withdrawing group.

Evidence that Heme of Green Hemoprotein is Distinct from
all Known Prosthetic Groups and Synthetic Hemes—The
prosthetic group of the green hemoprotein is clearly distinguished
by spectral properties and reactivity from protoheme, heme c,
the prosthetic group of lactoperoxidase (26), siroheme (27), and
all other hemes without carbonyl groups in conjugation with
the tetrapyrrole nucleus. Moreover, this prosthetic group is
distinguished from heme a, by pyridine hemochrome spectrum,
water solubility, chloroform insolubility, and paper chromatographic
properties of the heme, by the pyridine hemochrome spectrum of the heme oxime, by the paper chromatographic
properties of the formyl ester, and by the spectrum of the
porphyrin in nonpolar solvents. These same properties show
the heme to be distinct from synthetic mono- and diformyl-
protoporphyrins are spectrally similar to the tetrapyrroles derived
from this hemoprotein (see Tables I and II).

The spectral findings suggest the presence of one formyl
group and one weaker electron-withdrawing group. The isolated
prosthetic group is similar to formylvinyldeuteroheme in terms
of pyridine hemochrome spectra of the heme and heme oxime
(Table III). The neutral spectrum of the porphyrin derived from
the prosthetic group has absorbance maxima similar to those of
porphyrin isomers with one formyl and one vinyl group and also
to porphyrins with one formyl group and one acetyl group
(Table II). However, the "etio"-type spectrum (peak intensities of IV > III > II > I) shown by the porphyrin is in contrast to the
"oxorhodo" spectra (III > II > IV > I) of the 2-formyl-
6-vinyl and the 2-acetyl,6-formyl derivatives of deuteroporphyrin
II and the "rhodo" spectra (III > IV > II > I) of the 2-
formyl,4-vinyl and the 2-vinyl,4-formyl derivatives of deu-
teroporphylin IX. Of the model compounds with similar absorbance maxima reported in the literature, only 2-(4-formyl,
4(2)-acetyldeuteroheme IX (with its electron-withdrawing
groups on adjacent pyrroles) shows the same etio spectrum.
The data suggest that the prosthetic group under study posses-
ses one or more electron-withdrawing groups on the pyrrole
(or pyrroles) adjacent to the formyl-substituted pyrrole and that
the "rhodofying" effect of this group (or groups) must be greater
than that of a single vinyl group.

Presence on Heme of Acetylatable, Polar Group—Methyla-
tion converts the very water-soluble and chloroform-insoluble
prosthetic group into a methyl ester which is insoluble in water
and soluble in chloroform. As described above, the chromato-
grphic properties show that the methylated product contains
no free carboxyl group. However, the products resulting from
methylation with oxidinium salt, methanol/sulfuric acid, and
methanol/hydrochloric acid all migrate on paper chromatography in nonpolar solvents at a slower rate than the methyl esters
of protoheme, heme a, and 2-hydroxymethyl,4-vinyldeuteroheme (Table IV), indicating that the side chains on the methyl
ester of the prosthetic group have greater polarity than one
hydroxyl group. Chromatography in the propanol/kerosene
system shows that the methylation product resulting from
reaction with oxonium salt has greater polarity than the pro-
products of the two other methylation reactions. It would appear
that the acidic conditions of the methanol/sulfuric acid and
methanol/hydrochloric acid reactions result in the modification
or cleavage of a polar portion of the molecule.

*From this study.

small (Table III) and none of the known ketonic hemes and
porphyrins are spectrally similar to the tetrapyrroles derived
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prosthetic group are acetylatable and suggesting the presence
of multiple hydroxyl groups or possibly an amino group. How-
ever, even after the methylation-acetylation reaction sequence
the derivative is still a more polar compound than 2,4-diformyl-
deuteroheme as evidenced by its failure to migrate in the
chloroform/kerosene solvent. It is unclear whether this polar,
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water solubility, chloroform insolubility, and paper chromatographic
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properties of the formyl ester, and by the spectrum of the
porphyrin in nonpolar solvents. These same properties show
the heme to be distinct from synthetic mono- and diformyl-
protoporphyrins, synthetic mono- and diacetylporphyrins, and the cryptohemes \( p \) derived from protoporphine (28).

Whereas the prosthetic group is spectrally similar to Spiro-
graphis heme (2-formyl,4-vinyldeuteroheme IX), the two hemes
have very different chemical reactivities and polarity. In con-
TABLE II
Spectral properties of porphyrin derived from prosthetic group of green hemoprotein and model porphyrin compounds

<table>
<thead>
<tr>
<th>Porphyrin Derived from Prosthetic Group</th>
<th>Absorbance Maxima (nm) in Pyridine/Water (4:1)</th>
<th>Type Spectrum</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-Diformyldeuteroporphyrin IX Dimethyl Ester</td>
<td>426 521 558 589 646</td>
<td>Etio</td>
<td>a</td>
</tr>
<tr>
<td>Methyl Ester of Porphyrin Derived from Prosthetic Group</td>
<td>423 518 561 590 647</td>
<td>Etio</td>
<td>a</td>
</tr>
<tr>
<td>Protoporphyrin IX Dimethyl Ester</td>
<td>405 504 538 575 630</td>
<td>Etio</td>
<td>a</td>
</tr>
<tr>
<td>2,4-Diformyldeuteroporphyrin IX Dimethyl Ester</td>
<td>437 524 561 593 648</td>
<td>Etio</td>
<td>a</td>
</tr>
<tr>
<td>2,6-Diformyldeuteroporphyrin II Diethyl Ester</td>
<td>421 529 575 598 650</td>
<td>Oxorhodo</td>
<td>20</td>
</tr>
<tr>
<td>Porphyrin α Dimethyl Ester</td>
<td>418 520 563 584 646</td>
<td>Oxorhodo</td>
<td>21</td>
</tr>
<tr>
<td>Cryptoporphyrin α Dimethyl Ester</td>
<td>421 519 559 584 642</td>
<td>Rhodo</td>
<td>19</td>
</tr>
<tr>
<td>2-Formyl, 4-vinyldeuteroporphyrin IX Dimethyl Ester</td>
<td>420 519 559 584 642</td>
<td>Rhodo</td>
<td>21</td>
</tr>
<tr>
<td>2-Formyl, 6-vinyldeuteroporphyrin II Diethyl Ester</td>
<td>420 519 559 584 642</td>
<td>Rhodo</td>
<td>21</td>
</tr>
<tr>
<td>2(4)-Formyl, 4(2)-acetylideuteroporphyrin IX Dimethyl Ester</td>
<td>418 521 564 584 645</td>
<td>Oxorhodo</td>
<td>20</td>
</tr>
<tr>
<td>2-Acetyl, 6-formyldeuteroporphyrin II Diethyl Ester</td>
<td>521 561 587 646</td>
<td>Etio</td>
<td>22</td>
</tr>
<tr>
<td>2-Acetyl, 6-formyldeuteroporphyrin II Diethyl Ester</td>
<td>419 525 569 593 646</td>
<td>Oxorhodo</td>
<td>20</td>
</tr>
<tr>
<td>2-Formyl, 4-hydroxyethyldeuteroporphyrin IX Dimethyl Ester</td>
<td>516 556 581 641</td>
<td>Rhodo</td>
<td>22</td>
</tr>
<tr>
<td>2(4)-Formyldeuteroporphyrin IX</td>
<td>515 555 580 641</td>
<td>Rhodo</td>
<td>23</td>
</tr>
<tr>
<td>2,4-Diacetylideuteroporphyrin IX Dimethyl Ester</td>
<td>517 552 587 640</td>
<td>Etio</td>
<td>24</td>
</tr>
<tr>
<td>2,6-Diacetylideuteroporphyrin II Diethyl Ester</td>
<td>417 521 564 587 644</td>
<td>Oxorhodo</td>
<td>20</td>
</tr>
<tr>
<td>2(4)-Acetyl, 4(2)-vinyldeuteroporphyrin IX Dimethyl Ester</td>
<td>414 513 552 582 636</td>
<td>Etio</td>
<td>25</td>
</tr>
</tbody>
</table>

*From this study.

*We wish to thank Dr. Jack Barrett for his personal communication pointing out the spectral similarities of these hemes.

Contrast to the properties described in this paper, Spirographis heme does not show marked acid lability, does not possess an acetylatable polar group, and shows solubility typical of hemes in nonpolar solvents. Spirographis heme migrates faster on paper chromatography in the lutidine/water/ammonia system. The methyl ester of the Spirographis heme migrates much faster on paper chromatography in nonpolar solvents than does the methyl ester of the new prosthetic group. Moreover, the Spirographis porphyrin shows a "rhodo" spectrum while the porphyrin derived from the erythrocyte protein shows an "etio" spectrum with absorbance maxima at longer wavelengths.

Of interest is the spectral similarity between the spectral properties reported in this study and those reported² for cryptoheme a. Cryptoheme a was extracted from heart muscle prior to the isolation of heme a and was initially postulated by Negelein to be the prosthetic group of cytochrome oxidase (29). The isolation of cryptoheme a has been reported from several sources and by a number of laboratories but the significance of these findings has yet to be explained (19, 30–32). This compound appears to be a 2,4-substituted derivative of deuteroheme IX with a formyl group, an olefinic group, and a large molecular weight side chain (33). As shown in Tables I and III, cryptoheme a and the new heme from the erythrocyte protein show similar pyridine hemochrome spectra before and after oxime formation. However, in contrast to the properties of the new heme, cryptoheme a is soluble in diethyl ether, is stable in acid, migrates as a dicarboxylic tetrapyrrole, does not contain a polar side chain, and gives a porphyrin which shows a "rhodo" type spectrum (33). Thus, the heme we have isolated is not cryptoheme a but the hemes may have structural features.

²We wish to thank Dr. Jack Barrett for his personal communication pointing out the spectral similarities of these hemes.
TABLE III
Spectral properties of reduced pyridine hemochromes of oximes of formylhemes and acetylhemes

<table>
<thead>
<tr>
<th>Heme</th>
<th>Shift of a peak upon oxime formation</th>
<th>a Peak of oxime</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nm</td>
<td>nm</td>
<td></td>
</tr>
<tr>
<td>Prosthetic group of green hemo-protein</td>
<td>19</td>
<td>560</td>
<td></td>
</tr>
<tr>
<td>Heme a</td>
<td>17</td>
<td>570</td>
<td>23</td>
</tr>
<tr>
<td>2,4-Diformyldeuteroheme IX</td>
<td>21</td>
<td>563</td>
<td>23</td>
</tr>
<tr>
<td>2-formyl,4-vinyldeuteroheme IX</td>
<td>22</td>
<td>561</td>
<td>23</td>
</tr>
<tr>
<td>Cryptoheme a</td>
<td>20</td>
<td>562</td>
<td>19</td>
</tr>
<tr>
<td>2(4)-Formyldeuteroheme IX</td>
<td>18</td>
<td>560</td>
<td>23</td>
</tr>
<tr>
<td>2(4)-Acetyldeteroheme IX</td>
<td>2</td>
<td>569</td>
<td>23</td>
</tr>
<tr>
<td>2,4-Diacetyldeteroheme IX</td>
<td>2</td>
<td>570</td>
<td>23</td>
</tr>
</tbody>
</table>

TABLE IV
Rf values of heme methyl esters on paper chromatography in nonpolar solvents

<table>
<thead>
<tr>
<th>Heme</th>
<th>Methyla-</th>
<th>Chloroform</th>
<th>Propanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lating</td>
<td>form/</td>
<td>pro-</td>
</tr>
<tr>
<td></td>
<td>agent</td>
<td>kerosene</td>
<td>panol/kero-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1/1)</td>
<td>sene (9/50)</td>
</tr>
<tr>
<td>Prosthetic group</td>
<td>Me3OBF4</td>
<td>0.00</td>
<td>0.05</td>
</tr>
<tr>
<td>Acetic anhydride-treated prosthetic group</td>
<td>Me3OBF4</td>
<td>0.00</td>
<td>0.72</td>
</tr>
<tr>
<td>Prosthetic group</td>
<td>H2SO4/methanol</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>Prosthetic group</td>
<td>HCl/methanol/oxy-</td>
<td>0.06</td>
<td>0.67</td>
</tr>
<tr>
<td>Protoheme IX</td>
<td>Me3OBF4</td>
<td>0.89</td>
<td></td>
</tr>
<tr>
<td>Heme a</td>
<td>Me3OBF4</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>Heme a</td>
<td>H2SO4/methanol</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>2(4)-Hydroxymethyl,4(2)-vinyldeuteroheme IX</td>
<td>dimethyl ester</td>
<td>0.42</td>
<td></td>
</tr>
</tbody>
</table>

in common. Since acetic acid converts the prosthetic group to a derivative which then behaves on paper chromatography as a dicarboxylic heme, it is even possible that cryptoheme a is derived from this heme by the acid conditions of heme extraction.

We conclude that the prosthetic group of the erythrocyte hemoprotein contains one formyl group, a polar and acetylatable side chain, and either a ketonic or olefinic side chain. The best candidate for the polar side chain would be a moiety with multiple hydroxyl groups. The formyl group and the second electron-withdrawing side chain must be on adjacent pyrroles. Since all mammalian heme prosthetic groups studied to date are derivatives of deuteroheme IX substituted in the 2, 4, and, in some cases, the 8 position, we postulate that the heme under study has the structure

\[
\begin{align*}
R_1 & \quad \text{CH}_3 \\
R_2 & \quad \text{H}_2 \\
R_3 & \quad \text{CH}_3 \\
R_4 & \quad \text{CH}_2 \\
R_5 & \quad \text{CH}_2 \\
R_6 & \quad \text{COO}^- \\
R_7 & \quad \text{COO}^- \\
\end{align*}
\]

We further suggest as a working hypothesis that \( R_1 \) is a ketonic group, \( R_2 \) is a formyl group, and multiple hydroxyl groups are present on \( R_1 \) or \( R_2 \).

Acknowledgments—We wish to thank Dr. Louis DeFilippi and Mr. Richard Douglas for their ideas and participation in this study.

REFERENCES
Unique Prosthetic Group of Erythrocyte Hemoprotein

**EXPERIMENTAL PROCEDURE**

**MATERIALS**

Fish erythrocytes were obtained from the Freshwater Fish Research Center, University of Michigan, Ann Arbor, Michigan. The erythrocyte membranes were prepared as described by Karp and associates [4].

**METHODS**

Methods were adapted from those described by Karp and associates [4] and from those used by the author in previous studies [5].

**RESULTS**

The results obtained were consistent with those obtained by Karp and associates [4] and with those obtained by the author in previous studies [5].

**DISCUSSION**

The results obtained were consistent with those obtained by Karp and associates [4] and with those obtained by the author in previous studies [5].

**CONCLUSIONS**

The results obtained were consistent with those obtained by Karp and associates [4] and with those obtained by the author in previous studies [5].

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