# REDUCED UROPORPHYRIN III, A DIRECT INTERMEDIATE IN HEME BIOSYNTHESIS

by

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A THESIS

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(Chairman, Graduate Council)

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TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

# INTRODUCTION

## The Position of Porphyrins in Nature

Nowhere is the relationship between structure and function more adequately expressed than in the field of comparative biochemistry. The potential number of organic molecular structures appears to be almost infinite, yet nature has selected relatively few for reproduction by its living organisms. Analogy is drawn between this and the 26 letters of the alphabet whose possible numerical arrangements reach a staggering total. Nevertheless there is a finite number of possibilities with an even greater numerical limitation imposed by the process of communication. Experience has also shown that amino acid patterns have been limited to such an extent that all proteins may be classified within relatively few homologous structural categories (1).

The tetrapyrrole molecule is another example of a structural form which nature has seen fit to use repeatedly. In the Plant Kingdom, protoporphyrin with slight structural alteration, is found as the magnesium complex, chlorophyll, which functions in photosynthesis, an energy storing process. In the Animal Kingdom, energy yielding reactions are generally dependent on protoporphyrin as the iron complex which is coupled with specific proteins. These include the oxygen carriers hemoglobin and myoglobin, and several important enzymes, including catalase, the peroxidases, and the

cytochromes. The interrelationship is seen in the following equation: (2)

$$CO_2 + H_2O + \text{Energy} \xrightarrow[\text{Heme}]{\text{Chlorophyll}} O_2 + \text{Organic Compounds}$$

The important place of the tetrapyrroles in nature has focused a great deal of attention on their pathway of biosynthesis and the intermediate compounds between the initial glycine-succinate condensation and the monopyrrole, porphobilinogen, have been established. However, knowledge about multipyrrole formation, interconversion, and metal complexing of the tetrapyrroles has barely proceeded beyond the speculative stage.

The purpose of the work reported herein was to help elucidate these unknown factors in heme biosynthesis. The site of iron incorporation, long assumed to be protoporphyrin, is questioned, and a new tetrapyrrole intermediate in heme biosynthesis is described.

## Established Intermediates of Porphyrin Synthesis

Our knowledge about the mechanism of porphyrin synthesis has advanced markedly since the discovery of the first porphyrin in 1867 by Thudicum (3); Garrod's classification of porphyria as an inborn error of metabolism in 1923 (4); and Hans Fischer's synthesis of heme in 1929 (5). More recently major contributions have included

clinical studies (6, 7, 8, 9), the induction of porphyria in experimental animals by the administration of drugs (10, 11, 12), and in vitro studies using normal animal tissues, most notably the avian erythrocyte (13, 14, 15).

Biochemists in this country and in England have clarified the stepwise biosynthesis of protoporphyrin. Glycine and succinate have been shown to be direct precursors of heme (Fig. 1), and the source of each atom of protoporphyrin from these two precursors has been defined in elegant fashion (16). δ-Aminolevulinic acid (17) was proposed and later found to be an intermediate, as was the monopyrrole, porphobilinogen (18, 19, 20).

Quite recently, tentative pathways have been suggested by Shemin, and Bogorad and Granick for the conversion of the monopyrrole to the tetrapyrrole form (21, 22). Shemin has postulated that a reduced tetrapyrrole of the type III configuration might be formed by the coupling of two dissimilar dipyrroles arising from porphobilinogen. The mechanism of the formation involves the condensation of three moles of porphobilinogen leading to a tripyrrylmethane which splits to give a dipyrrylmethane and a monopyrrole (Fig. 2). The structure of the dipyrrylmethane is dependent upon the point of cleavage. A split at A gives dipyrrylmethane A, and at B, dipyrrylmethane B. Condensation of the dipyrrylmethane molecules in the following manner would yield a porphyrin

**Figure 1**

**The reaction sequence leading from succinate and glycine to porphobilinogen, the monopyrrole precursor of porphyrins (2).**

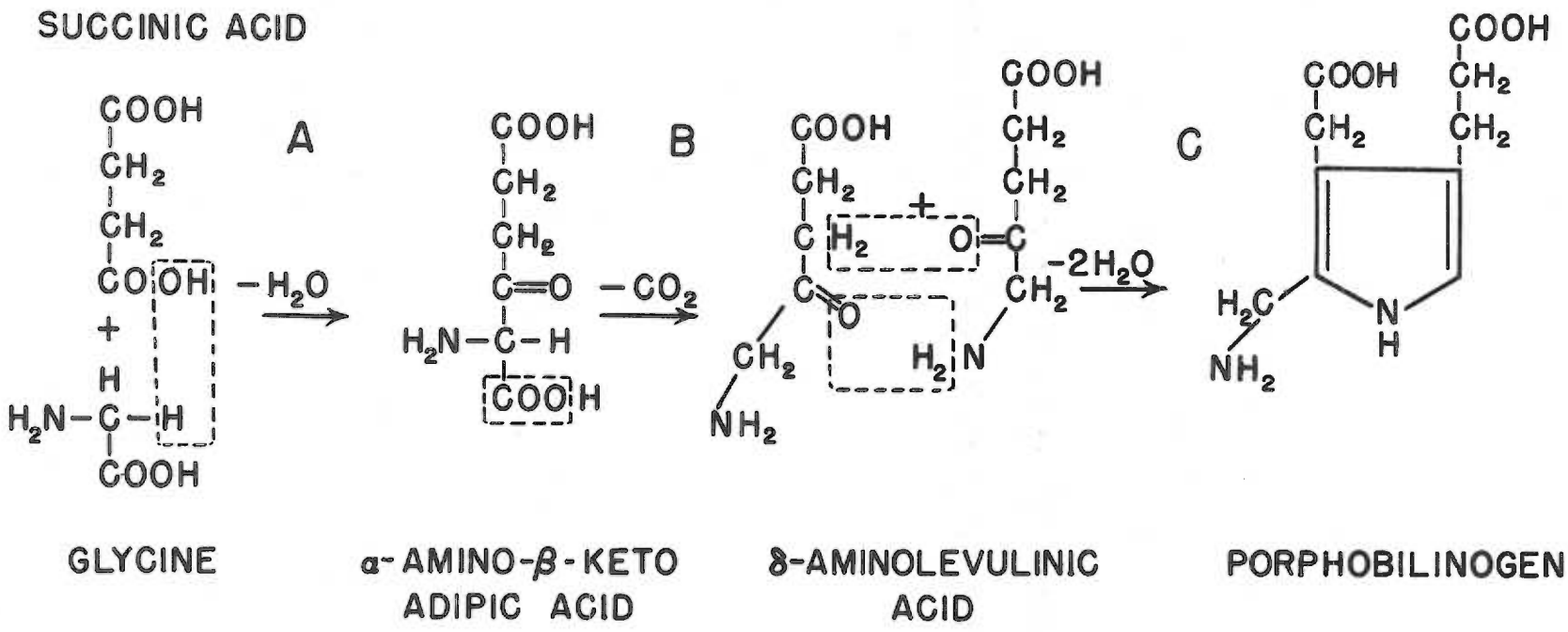
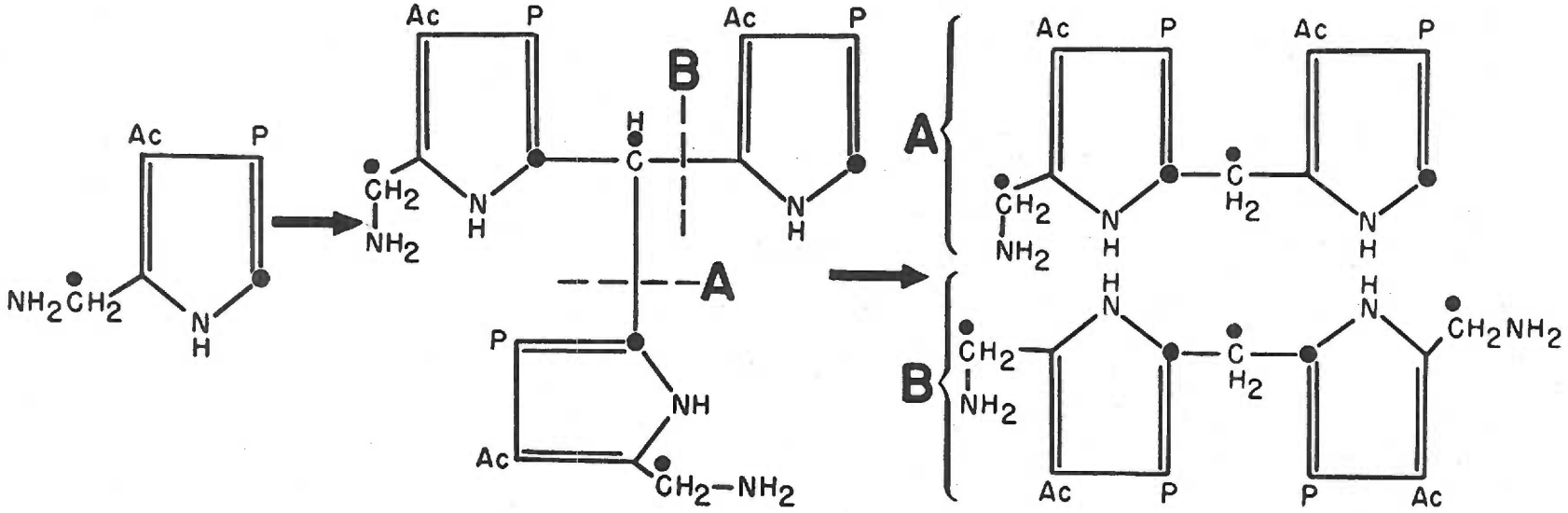

SUCCINIC ACID
A
B
C
$-H_2O$
$-CO_2$
$-2H_2O$
GLYCINE
α-AMINO-β-KETO ADIPIC ACID
δ-AMINOLEVULINIC ACID
PORPHOBILINOGEN

**Figure 2**

**A mechanism of porphyrin formation from the monopyrrole.**




A mechanism of porphyrin formation from the monopyrrole. Ac = acetic acid side chain; P = propionic acid side chain; ● = $\alpha$-carbon atom of glycine and $\delta$-carbon atom of $\delta$-aminolevulinic acid.

From: Shemin, D.; J. Biol. Chem. 215, 613, 1955.

of the I or III series; two moles of A yield a series I tetrapyrrole; while one mole of A and B each yields a series III tetrapyrrole. Two moles of B cannot condense because there is no carbon available to form a methene bridge. It should be noted in view of data presented subsequently, that the condensation product of a type A and type B dipyrryl derivative would be a reduced derivative of uroporphyrin III.

## Tetrapyrroles Investigated as Heme Intermediates

Any consideration of possible heme intermediates must necessarily include uroporphyrin, coproporphyrin, and hematoporphyrin because of their structural similarity to protoporphyrin (Fig. 3). One may logically question whether or not these substances have any function, for there is little evidence that uroporphyrin, coproporphyrin, or hematoporphyrin have any physiological significance. For that matter, protoporphyrin must also be included in this category because its functional capacity is dependent upon the presence of a metal, and of course the specific protein to which it is attached. A plausible function for these molecules, including their open chain derivatives, is that of an intermediate or end product somewhere in the sequence of heme formation or breakdown.

## Hematoporphyrin

Early workers in porphyrin chemistry considered the urinary porphyrins to be hemoglobin breakdown products primarily because

**Figure 3**

**Structural Formulae of Porphyrin**

**Legend:**

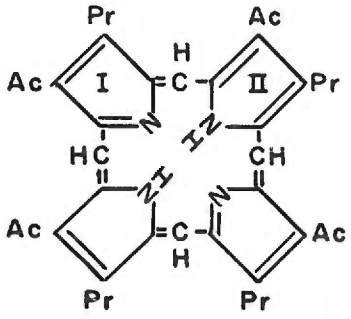
Ac = $-CH_2-CO_2H$

Pr = $-CH_2-CH_2-CO_2H$

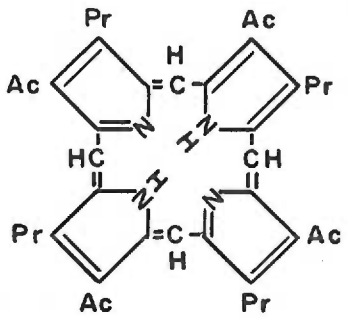
M = $-CH_3$

V = $-CH=CH_2$

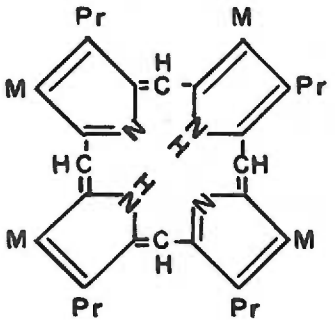
OH-ET = $-CH(OH)-CH_3$



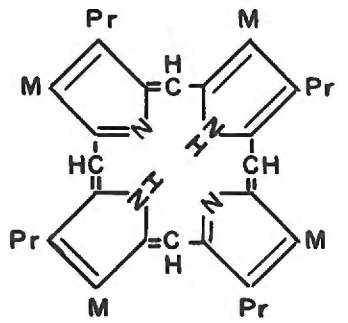

UROPORPHYRIN III



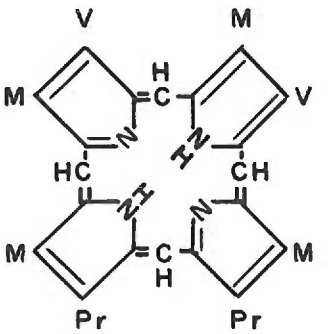

UROPORPHYRIN I



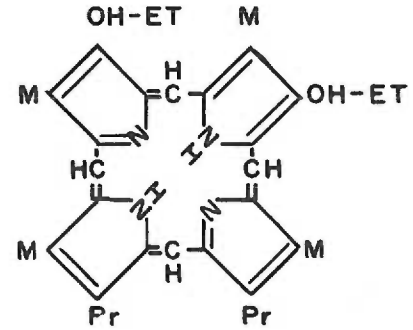

COPROPORPHYRIN III




COPROPORPHYRIN I




PROTOPORPHYRIN 9




HEMATOPORPHYRIN 9

of the influence of Hoppe-Seyler, hence the term "hematoporphyrin". He also produced the porphyrin chemically from hemoglobin with acid (23). Fischer (24) demonstrated that hematoporphyrin was a separate entity and could be distinguished from the urinary porphyrins. He further stated that this molecule was not found in nature but was a result of chemical rather than biochemical processes. In recent years, evidence has accumulated to imply that hematoporphyrin may be a precursor of protoporphyrin because of the high concentration found in a Chlorella mutant which was also producing protoporphyrin (25). Eriksen (26), studying the effect of lead on heme biosynthesis, demonstrated a dicarboxylic porphyrin different from protoporphyrin and suggested that it might be hematoporphyrin. He relegated to it the position of an intermediate in the formation of the free erythrocyte protoporphyrin and thus a precursor of heme. In a recent investigation, incubation of $C^{14}$ labeled hematoporphyrin with bone marrow homogenate yielded protoporphyrin containing 5 per cent of the total added activity (27). This would seem to be negligible for such a proximate intermediate unless the equilibrium was far in the direction of hematoporphyrin. The evidence thus far appears more suggestive than convincing in designating hematoporphyrin as a true heme precursor.

Uroporphyrin

Neuberger (28) and Radin and Shemin (29) independently sug-

gested that the synthesis of heme proceeds through a progressive decarboxylation and dehydrogenation of uroporphyrin and coproporphyrin to protoporphyrin. This is of interest when one considers that only recently has the presence of uroporphyrin been confirmed in normal human urines (30, 31) and in the erythrocytes of patients with hemoglobin aberrations (32). These new facts are the result of improved laboratory techniques. Earlier identification of urinary uroporphyrin was confined to clinical cases of porphyria which manifested themselves by large outpourings of this fluorescent pigment. Because of its structural similarity to protoporphyrin it logically was considered as a heme precursor which, under normal conditions, was very likely present only briefly, since it underwent rapid transformation to protoporphyrin or hemoglobin. On the basis of experimental evidence the position of uroporphyrin as a heme precursor is tenuous, for in only one instance (33) has it been experimentally cited as an intermediate, a finding often tested and as yet unconfirmed. The strongest evidence against this hypothesis was obtained by Schwartz (34). He incubated $C^{14}$ labeled uroporphyrin with an activity of 190,000 counts per minute per milligram with liver homogenates and bone marrow; perfused the same material through liver; and even injected it into rats and rabbits, but the isolated coproporphyrin and protoporphyrin failed to show any activity.

Coproporphyrin

Studies of coproporphyrin in normal urine and blood and in a

variety of clinical conditions have provided valuable information about this tetrapyrrole. Early studies of hemopoiesis by Dobriner (35) indicated that increased coproporphyrin I excretion parallels reticulocyte formation. He also suggested that the coproporphyrin was merely a side product in heme formation because the type I isomer was considered predominant while protoporphyrin is type III. Watson, at one time, held that the coproporphyrin I isomer was predominant and that coproporphyrin III was not a normal product of metabolism, but rather: 1) a derivative from hemoglobin in the diet; 2) a resultant of lead poisoning; or 3) due to abnormal conditions in the liver resulting from alcoholism (36). Such an hypothesis requires the breaking of the porphyrin ring and rearrangement of the pyrroles, a highly unlikely possibility in view of later work. Schwartz (37) more recently has identified the normal erythrocyte coproporphyrin as the type III isomer, thus supporting the contention of Rimington (38) that porphyrin synthesis proceeds mainly in the direction of the type III isomer.

Using $N^{15}$ labeled glycine, Lowry (39) found a similarity in the $N^{15}$ curves of hemoglobin and fecal coproporphyrin and protoporphyrin, suggesting that their formation might be related to the synthesis of hemoglobin. These experiments also thoroughly disposed of the possibility of coproporphyrin or protoporphyrin arising from the catabolism of hemoglobin as suggested by Van den Bergh (40)

and Grinstein (41). With the breakdown of hemoglobin there is a rise in the $N^{15}$ content of stercobilin. If either porphyrin were the result of this catalytic process then a rise in their $N^{15}$ concentration should have occurred.

Using a different approach to study heme biosynthesis, Yeas and Starr (42) measured catalase, cytochrome c, and hematin production in a yeast strain deficient in its ability to synthesize glycine. Addition of either glycine or protoporphyrin resulted in formation of catalase, cytochrome c, and hematin. Coproporphyrin III was ineffective as a precursor to protoporphyrin with a consequent decrease in hematin and the porphyrin enzymes, catalase and cytochrome c. This is in agreement with the more recent work of Shemin (22) who noted that $C^{14}$ labeled coproporphyrin III is not converted to heme in the duck erythrocyte preparation. In addition, Schwartz (34) has also failed to find conversion of labeled coproporphyrin III to heme as noted in experiments similar to those described under uroporphyrin.

Protoporphyrin

Further progress toward a complete explanation of heme biosynthesis has been hampered by a lack of information about the biochemical mechanism for combining iron and a specific protein with the final functional tetrapyrrole, protoporphyrin. Evidence for a relationship of protoporphyrin to heme synthesis is apparent from the work of several investigators. However, there is no unequivocal

experimental evidence to show that iron can be directly introduced into protoporphyrin in any biological system to form heme. Granick (43) has previously reported that chicken erythrocytes will enzymically form heme from iron and protoporphyrin. Later, in a personal communication he stated that these studies were inconclusive. Schwartz (34) incubated $C^{14}$ labeled protoporphyrin with bone marrow and liver homogenate and found a small amount of activity in the isolated heme. More recently, Goldberg (44) reported $Fe^{59}$ incorporation into protoporphyrin to a greater degree than that found by Schwartz and Granick.

In an excellent series of experiments, Dresel (45) tested porphobilinogen, δ-aminolevulinic acid, uroporphyrin III, coproporphyrin III, hematoporphyrin 9 and protoporphyrin 9 as glycine diluents. Glycine is an essential precursor in the biosynthesis of heme, and therefore one might expect a diminution of heme radioactivity from glycine-2-$C^{14}$ in the presence of other non-labeled direct intermediates. Her results indicated that only porphobilinogen and δ-aminolevulinic acid diluted the glycine-2-$C^{14}$ sufficiently to produce the diminution in heme activity expected of a direct intermediate. Significantly protoporphyrin could not be included. It is therefore unlikely that protoporphyrin is the principal iron binding intermediate in heme synthesis.

The experiments presented in this thesis were designed to

determine whether iron is incorporated into protoporphyrin. The results strongly indicate that protoporphyrin is not the principal iron acceptor. Structurally related pyrroles were tested and these were also shown not to be directly involved. Therefore, tetrapyrroles, whose existence in the reduced state has been suspected, were sought between porphobilinogen and heme. Chemical reduction of known porphyrins to the "pro-porphyrins" or autoxidisable porphyrinogens was accomplished. These reduced derivatives were then tested and one of them shown to be a heme intermediate. The results of these experiments are described in the following pages.

# MATERIALS AND METHODS

## A. The Lysed Duck Erythrocyte System

The lysed avian erythrocyte system has been used extensively in studying the position of glycine and succinic acid, δ-amino-levulinic acid, and porphobilinogen in the pathway of heme biosynthesis. Lysed duck red blood cells were therefore considered to be a suitable medium for the study of intermediates appearing after porphobilinogen in the sequence of heme formation.

Blood was obtained from the jugular vein of White Peking or Muscovy (cairina moschata) ducks and prepared according to the method described by Shemin and Kumin (13). The heparinized blood is centrifuged and the plasma removed. The red cells are washed four times with isotonic saline and then suspended in a volume of isotonic sucrose equal to the original volume of plasma. When pooling of blood from several ducks is necessary, the cells are first washed with the isotonic saline. Incubation of the erythrocytes for one hour at room temperature in isotonic sucrose allows the system to use up most of the endogenous porphyrin precursors. After incubation, the cells are separated from the sucrose by centrifugation and lysed by the addition of one and one half volumes of cold water. The lysed blood is then divided into 20 ml. aliquots for further incubation with the particular test substrate. The duration and temperature of the incubation period for each experiment

are cited at the bottom of the respective Table.

In some experiments the lysed cell preparation was homogenized in a Waring Blendor fitted with a water jacket to prevent any marked increase of temperature during the 10 minute homogenization period. The purpose of this procedure is to destroy the glycine-succinate coupling enzyme (46), thus reducing the amount of porphyrin and heme which might be synthesized from these common endogenous precursors. The use of this technique is indicated in the appropriate experiments.

B. Solubilization of the Porphyrins for use in the Duck System

A factor of considerable annoyance was the low solubility of the porphyrins at physiologic pH. At high or low pH, solubility was no problem, but the acid or alkaline solution destroyed or inhibited the enzyme systems involved. It was found that with careful addition of sodium bicarbonate to the acid solutions (HCl), neutralization could be affected without crystallization of the porphyrins. This solution, however, was unstable and precipitation usually occurred after several hours. Conversely, an alkaline solution (KOH) of the porphyrins could be neutralized to pH 7.6 by the careful addition of dilute acid (1.5 N HCl). Below this pH crystallization occurred. The porphyrins may be ranked empirically according to their relative ease of solubilization. Uroporphyrin was the easiest and protoporphyrin the hardest to get into aqueous solution. The difficulties with protoporphyrin were somewhat alleviated by the observation that

M/7 sodium bicarbonate was a better solvent. Hematoporphyrin was found to be soluble in dilute sodium citrate, and did not precipitate as easily at physiologic pH as did the other porphyrins.

C. Preparation of Porphyrins from Hemin, Erythrocytes, and Urine

1) Preparation of Protoporphyrin from Hemin (47)

Protoporphyrin was prepared by a modification of the method of Fischer and Putzer (48). Six gm. of recrystallized hemin and 400 grams of 95 per cent formic acid are added to a one liter 3 necked flask, with stirrer and reflux attached and heated on an oil bath. A total of 6 gm. of reduced iron powder is added in the following way. Before the formic acid comes to a boil, 0.3 gm. of iron is added. As boiling begins, the iron is added in 6 to 7 portions over a period of 20 to 25 minutes, with an additional 5 minutes of boiling at the end. The flask is removed from the bath and after 10 minutes the contents are filtered by suction, and the residue on the filter paper washed with a little formic acid. The deep red formic acid solution is poured into 2 liters of water. Then 100 cc. of saturated ammonium acetate solution is added on the side of the beaker without stirring and left overnight at room temperature. The fine flocculent protoporphyrin is centrifuged, and then transferred to a suction filter, washed, and dried in vacuo and recrystallized.

2) Preparation of Protoporphyrin from Erythrocytes (49)

Whole blood, or clotted blood (25-50 ml.) are mixed with 2-3 volumes of acetone. The resultant precipitate is filtered through a

Buchner funnel and washed with acetone 2 or 3 times. Suction is applied until a dry powder (mostly hemoglobin) is obtained (grayish in color). The powder is suspended in 10 per cent oxalic acid in methanol and shaken for 10 minutes. The hematin solution is separated by filtration using a Buchner funnel and #5 Whatman filter paper. Extraction is repeated with several portions of oxalic acid solution until the extracted solution is colorless (4-5 extractions). Three to five grams of $FeSO_4$ per 100 ml. are dissolved in the combined hematin solutions. Precipitation may take place here. The solution is then saturated with a strong current of gaseous HCl (produced from NaCl plus concentrated $H_2SO_4$). The hematin solution becomes clear and the temperature rises. The color changes to a red brown then to a violet. Protoporphyrin has become esterified at this point and the HCl may be stopped. The solution is mixed with chloroform and a large excess of water and allowed to stand several minutes until the ester is concentrated in the chloroform. The latter solution is washed three times with distilled water, once with 10 per cent ammonia solution, and three times again with distilled water. A white precipitate sometimes forms with the ammonia wash and should be removed. The final chloroform solution is concentrated and mixed with methanol to give a precipitate of the methyl ester of protoporphyrin. The free protoporphyrin is prepared by saponification of the ester in 25 per

cent HCl for 4-5 hours and is precipitated by neutralization with dilute NaOH.

3) Preparation of Uroporphyrin and Coproporphyrin from Urine of Porphyric Patients (50)

Urine was obtained from patients with acute porphyria and from rabbits made porphyric with AIA (allyl-isopropyl-acetamide) (51). At pH 4-6 coproporphyrin is extracted into ethyl acetate, and uroporphyrin remains in the aqueous phase. Removal of coproporphyrin from the ethyl acetate is accomplished by the addition of small amounts of 1.5 N HCl. Buffering this solution again to a pH of 3.2 with a saturated solution of sodium acetate and extracting the coproporphyrin with ether results in a highly purified product which is removed from the ether by the addition of 1.5 N HCl.

Aluminum oxide (5 gm./40 cc.) is added to the aqueous phase remaining after coproporphyrin extraction. The slurry is well mixed and then allowed to settle (aided by centrifugation). The uroporphyrin is absorbed on the aluminum oxide and, after washing with water, can be removed by the addition of 1.5 N HCl.

Esterification of the porphyrins proceeds readily in ten volumes of methanol-sulfuric acid (20:1 by volume).

4) Preparation of Uroporphyrin from the Wing Feathers of Turacus hartlaubi (52)

The wing feather is cut into small pieces and placed in a beaker of 0.1 N KOH. Gentle agitation quickly and efficiently removes the

Cu-uroporphyrin complex, which is precipitated by the addition of several drops of concentrated glacial acetic acid. The precipitated Cu-complex is washed several times with water and redissolved in 0.1 N KOH.

Reduction of the Cu-complex with sodium amalgam removes the Cu, leaving reduced uroporphyrin in solution. Aeration of the reduced uroporphyrin solution for several hours results in reoxidation of the porphyrin, which is then precipitated with glacial acetic acid.

D. Preparation of Reduced Porphyrins

The amount of porphyrin required for each experiment was usually less than five milligrams. Therefore, a rough estimate of this amount of the porphyrin ester was weighed out. The ester is hydrolyzed in 3 N HCl for several hours and the free porphyrin precipitated by the addition of NaOH. The precipitate is washed with water twice and dissolved in a small volume (3-5 ml.) of 0.1 N KOH. Solubilization in a dilute alkaline medium is preferred since the reduction process with sodium amalgam was more easily carried out.

Quantitation by fluorimetry is made at this point. An extremely small volume of the alkaline porphyrin solution, usually 0.01 ml., is removed and diluted to 25 ml. with 1.5 N HCl. The concentration in this volume is compared to a standard coproporphyrin also in 1.5 N HCl. Coproporphyrin is the reference standard for all porphyrin fluorescence measurements because of its stability.

To the alkaline porphyrin solution is added a small amount of ammonium chloride (40 mg./ml.), and ascorbic acid (3 mg./ml.). Two to three grams of sodium amalgam (4-5 per cent) is added and the solution shaken until there is no visible fluorescence. The ascorbic acid retards the otherwise rapid reoxidation of the porphyrins and the ammonium chloride yields ammonia gas on the addition of the amalgam. This gaseous ebullition increased the surface area of the amalgam by producing a spongy mass and thereby greatly facilitated the reduction process. Reduction under these conditions is accomplished in 3-4 minutes. The solution is then filtered through a fine sintered glass filter, carefully adjusted to pH 7.6 with dilute HCl, and the volume adjusted (5-10 ml.). In the experiments with reduced porphyrins the abbreviation (max. amt.) is used. This refers to the maximum amount of reduced porphyrin available for assimilation. Experimentally the maximum amount equals about 25 per cent of that cited in each table (cf. Table XIV). The solution of reduced porphyrin is now ready for addition to the appropriate flask for incubation with the lysed duck erythrocyte cell preparation.

E. Isolation and Preparation of Hemin for Determination of Radioactivity (53)

In all the experiments approximately 1 μc of $Fe^{59}$ is used per 20 ml. of blood. The iron is added to the pooled lysed blood to insure

uniform distribution of the isotope. Individual samples are then transferred to the incubation flasks containing the test substrate. After incubation, the hemin is prepared by a modification of Fischer's method.

Three volumes of glacial acetic acid (containing 1 ml. of saturated sodium chloride) per volume of blood is brought to boiling, the blood added slowly, and boiling continued for 5 minutes with constant stirring. The blood-acetic acid mixture is digested on the steam bath for one hour, after which time the hemin is centrifuged while hot to minimize protein contamination. The precipitate is washed 2 times each with fifty per cent acetic acid, water, ethanol, ether and then dried. To obtain pure crystals, the heme is recrystallized. One half ml. of pyridine is added to the dry hemin crystals and triturated well for one minute. Chloroform (8-10 ml.) is added and the mixture is filtered. The filter paper is washed with chloroform (about 20 ml.) until colorless.

Approximately 15 ml. of glacial acetic acid is added and the mixture allowed to stand for 30 minutes. The solution is then heated to 105 °C. to remove the chloroform, thus allowing crystallization to take place in the concentrated acetic acid. A drop of concentrated HCl seems to aid crystallization.

The crystals are washed with acetic acid, water, ethanol, and ether as before. An appropriate amount of hemin is weighed out to

make a solution having a concentration of one milligram per milliliter. The solvent was pyridine or 1 N KOH. Two milliliter aliquots were routinely used. Purity of the radioactive hemin is indicated by the fact that a second recrystallization produced no change in the specific activity (Table I).

$Fe^{59}$ activity was measured in a Nuclear well type scintillation counter attached to a Berkeley Decimatic Scaler. $C^{14}$ activity of dry hemin crystals was measured with an end window Geiger-Muller tube and Berkeley Decimatic Scaler.

F. Quantitation of Porphyrins

1) Erythrocyte Coproporphyrin and Protoporphyrin

This procedure is a modification by Schwartz (54) of the method of Grinstein and Watson (55) and permits analysis of coproporphyrin and protoporphyrin. The centrifuged cells (or lysed cell preparation) are added to 10 volumes of a 4:1 mixture of ethyl acetate and glacial acetic acid. The suspension is thoroughly ground and the extraction repeated until the filtrate is essentially colorless. The porphyrin is removed from the ethyl acetate-acetic acid solution by exhaustive extraction with 3 N HCl.

The HCl solution is neutralized to Congo red with saturated sodium acetate and extracted with ethyl acetate. Coproporphyrin is removed from the ethyl acetate by the addition of 0.1 N HCl. The

TABLE I

Specific Activity of Hemin Samples on 1st and 2nd Recrystallization

| Hemin Activity (CPM) | |
|---|---|
| 1st Recrystallization | 2nd Recrystallization |
| 1024 | 930 |
| 468 | 440 |
| 840 | 890 |
| 990 | 960 |

Concentration of hemin in 1:0 N KOH = 1 mg./ml.

Volume of aliquot = 2 ml.

protoporphyrin is removed by addition of 3 N HCl. A sufficient volume of 7.5 N HCl is added to 0.1 N HCl-coproporphyrin solution to raise the normality to 1.5. The protoporphyrin solution is diluted with an equal volume of water. The solutions are then quantitated by fluorescence measurement. The absorbed ultraviolet radiation of porphyrins is re-emitted as light of longer wave length in the red region.

2) Erythrocyte uroporphyrin

This method is applicable only to those experiments in which uroporphyrin and reduced uroporphyrin were added. A 5 ml. aliquot of incubation mixture is extracted with an ethyl acetate-acetic acid mixture at pH 3.5. At this pH the uroporphyrin is very soluble in the acetic acid and can be extracted. Coproporphyrin is also extracted at this point. The porphyrins are removed from the ethyl acetate-acetic acid solution with 0.1 N HCl.

The HCl solution is neutralized to Congo red with saturated sodium acetate and is extracted with ether to remove coproporphyrin. The aqueous phase containing uroporphyrin is extracted with ethyl acetate. The uroporphyrin is then removed from the ethyl acetate with 1.5 N HCl and is quantitated fluorimetrically.

G. Other Materials

Hematoporphyrin hydrochloride

Purchased from Mann Research Laboratories Inc. New York 6, New York.

Ferrous citrate - $Fe^{59}$

Purchased from Abbott Laboratories, North Chicago, Illinois.

Glycine-2-$C^{14}$

Purchased from Nuclear Laboratories, Chicago, Illinois.

Porphobilinogen

Obtained from Dr. Samuel Schwartz

University of Minnesota Medical School

δ-Aminolevulinic acid hydrochloride

Generously supplied by Dr. John R. Dice, Research Department, Parke, Davis and Company, Detroit, Michigan

Wing feathers from Turacus birds

Generously supplied by:

Kenton C. Tint, Curator of Birds, Zoological Society of San Diego, California.

John Tee-Van, Director

New York Zoological Society, New York

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National Zoological Park, Washington, D. C.

# RESULTS

Before attacking the main problem of heme biosynthesis, preliminary experiments were designed to obtain some working information concerning the lysed duck erythrocyte system. Thus, one of the first points to be determined was the content in normal duck erythrocytes of the principal free porphyrin, protoporphyrin. This value was found to be about 40-70 μg per cent as seen from Tables II and III.

Investigation of the capacity of the system to synthesize protoporphyrin from established heme intermediates revealed that glycine and succinic acid were capable of producing about twice the original amount of free protoporphyrin during three hours of incubation (Table II). In the absence of these two intermediates, no protoporphyrin was produced during the same and longer time intervals of incubation (Table II). This suggests that the quantity of precursors normally present in the system is extremely small.

The assumption that iron is incorporated directly into protoporphyrin was tested by adding protoporphyrin and radioactive iron to the lysed duck cell preparation and measuring simultaneously the disappearance of protoporphyrin and the radioactivity of the resultant heme. The results in Table III show that the synthesis of heme as measured by $Fe^{59}$ uptake bears no correlation to the free protopor-

TABLE II

Coproporphyrin and Protoporphyrin

Synthesized by Lysed Duck Erythrocytes

| Time of Incubation (hrs) | Coproporphyrin (µg%) | | Protoporphyrin (µg%) | |
|---|---|---|---|---|
| | Glycine and Succinic Acid | | | |
| | with | without | with | without |
| 0 | 5.0 | | 40 | |
| 3 | 5.0 | 2.5 | 86 | 56 |
| 6 | 13.0 | 3.0 | 128 | 42 |
| 9 | 11.0 | 2.0 | 200 | 50 |
| 12 | 22.0 | 3.0 | 320 | 44 |

Glycine and succinic acid (0.5 µ Mole each per sample)

Temperature 37 ° C.

## TABLE III

### Correlation of $Fe^{59}$ Uptake and Protoporphyrin Disappearance During Heme Biosynthesis

| Atmosphere | Protoporphyrin (µg%) | | | Hemin Activity (CPM) |
|---|---|---|---|---|
| | 0 hrs. | 6 hrs. | Diff. | |
| Nitrogen | 75 * | 117 | + 42 | 2550 |
| | 135 ** | 142 | + 7 | 3870 |
| | 315 ** | 167 | -148 | 2430 |
| Air | 75 * | 43 | - 32 | 3930 |
| | 135 ** | 275 | +140 | 5050 |
| | 315 ** | 142 | 173 | 2350 |

* Endogenous protoporphyrin

** Endogenous plus added protoporphyrin

phyrin disappearance. Occasionally, with relatively high levels of added protoporphyrin (250-300 μg) there was a decreased recovery of protoporphyrin after incubation, but this again was not accompanied by a corresponding uptake of $Fe^{59}$ into heme (Table III). This finding suggested that iron was not bound under aerobic or anerobic conditions to the protoporphyrin moiety either by chemical chelation or enzymatic insertion. Similar findings were noted with very low levels of protoporphyrin (Table IV).

In contrast to protoporphyrin, there was produced a progressive increase in $Fe^{59}$ labeled heme upon the addition of glycine and succinic acid, δ-aminolevulinic acid, and porphobilinogen respectively (Table V).

Other tetrapyrroles (uroporphyrin, coproporphyrin, and hematoporphyrin) were tested in a systematic screening and yielded results identical to those found with protoporphyrin. These negative results were obtained repeatedly and, therefore, to avoid redundancy are illustrated only once and in conjunction with other results (Tables VI, VII, and VIII).

Since the common tetrapyrroles could not be established as direct heme intermediates by virtue of iron acceptance, attention was turned to the reduced derivatives of the respective porphyrins. Each porphyrin was tested in the oxidized and reduced state. The results are as noted:

## TABLE IV

### $Fe^{59}$ Uptake With Added Protoporphyrin During Heme Biosynthesis

| Protoporphyrin µg | Protoporphyrin µ Moles | Hemin Activity (CPM) |
|---|---|---|
| 0.0 | 0 | 2450 |
| 0.5 | 0.001 | 2460 |
| 1.0 | 0.002 | 2470 |
| 2.0 | 0.004 | 2430 |
| 5.0 | 0.01 | 1990 |
| 10.0 | 0.02 | 2490 |
| 20.0 | 0.04 | 2320 |

Incubation 6 hrs. at 37° C.

## TABLE V

$Fe^{59}$ Incorporation into Heme with Established Porphyrin Precursors

| Substrate | Micromoles | Hemin Activity | |
|---|---|---|---|
| | | CPM/μMole | CPM/mg |
| succinic acid and glycine | 170 | 3 | 515 |
| δ-aminolevulinic acid | 7.65 | 64 | 480 |
| porphobilinogen | 0.68 | 625 | 420 |

Incubation: 6 hrs. at 37° C.

## TABLE VI

### $Fe^{59}$ Uptake with Added Reduced Protoporphyrin

### During Heme Biosynthesis

| Reduced Protoporphyrin µg/sample (max. amt.) | Hemin Activity (CPM) |
|---|---|
| 0 | 1260 |
| 43 | 1400 |
| 86 | 1440 |
| 172 | 1160 |
| 258 | 940 |
| **Protoporphyrin (µg)** | |
| 52 | 1200 |
| 104 | 1240 |

Incubation: 6 hrs. at 37° C.

## TABLE VII

### $Fe^{59}$ Uptake with Added Reduced Hematoporphyrin During Heme Biosynthesis

| Reduced Hematoporphyrin (μg / sample) (max. amt. ) | Hemin Activity (CPM) |
|---|---|
| 0 | 2490 |
| 10 | 3150 |
| 25 | 3100 |
| 100 | 3000 |
| 400 | 2740 |
| 1000 | 2470 |
| **Hematoporphyrin** | |
| 10 | 2940 |
| 25 | 2950 |
| 100 | 2900 |
| 400 | 2680 |
| 1000 | 2930 |

Incubation: 6 hrs. at 37° C.

## TABLE VIII

### $Fe^{59}$ Uptake with Added Reduced Uroporphyrin III

### During Heme Biosynthesis

| Reduced Uroporphyrin III (μg / sample) (max. amt. ) | Hemin Activity (CPM) |
|---|---|
| 0 | 1360 |
| 70 | 2600 |
| 140 | 3100 |
| 280 | 3500 |
| **Uroporphyrin III (μg)** | |
| 0 | 1360 |
| 70 | 2120 |
| 140 | 1890 |
| 280 | 2120 |

Incubation: 6 hrs. at 37° C.

a) Protoporphyrin: As already seen, this porphyrin does not accept $Fe^{59}$. In the reduced form similar results are also noted (Table VI).

b) Hematoporphyrin: The results show no significant uptake of $Fe^{59}$. Reduced hematoporphyrin was likewise ineffective (Table VII).

c) Uroporphyrin: Uroporphyrin III showed no uptake of $Fe^{59}$. However, the reduced derivative produced incorporation of the tracer iron into heme (Table VIII). Later experiments (Fig. 4, Table IX) show this incorporation even more significantly.

To obtain a better supply of the pure isomer, uroporphyrin III was isolated from the wing feather of the Turacus bird (cf. methods). This bird is in the unique position of having the only known source of pure uroporphyrin isomer type III.

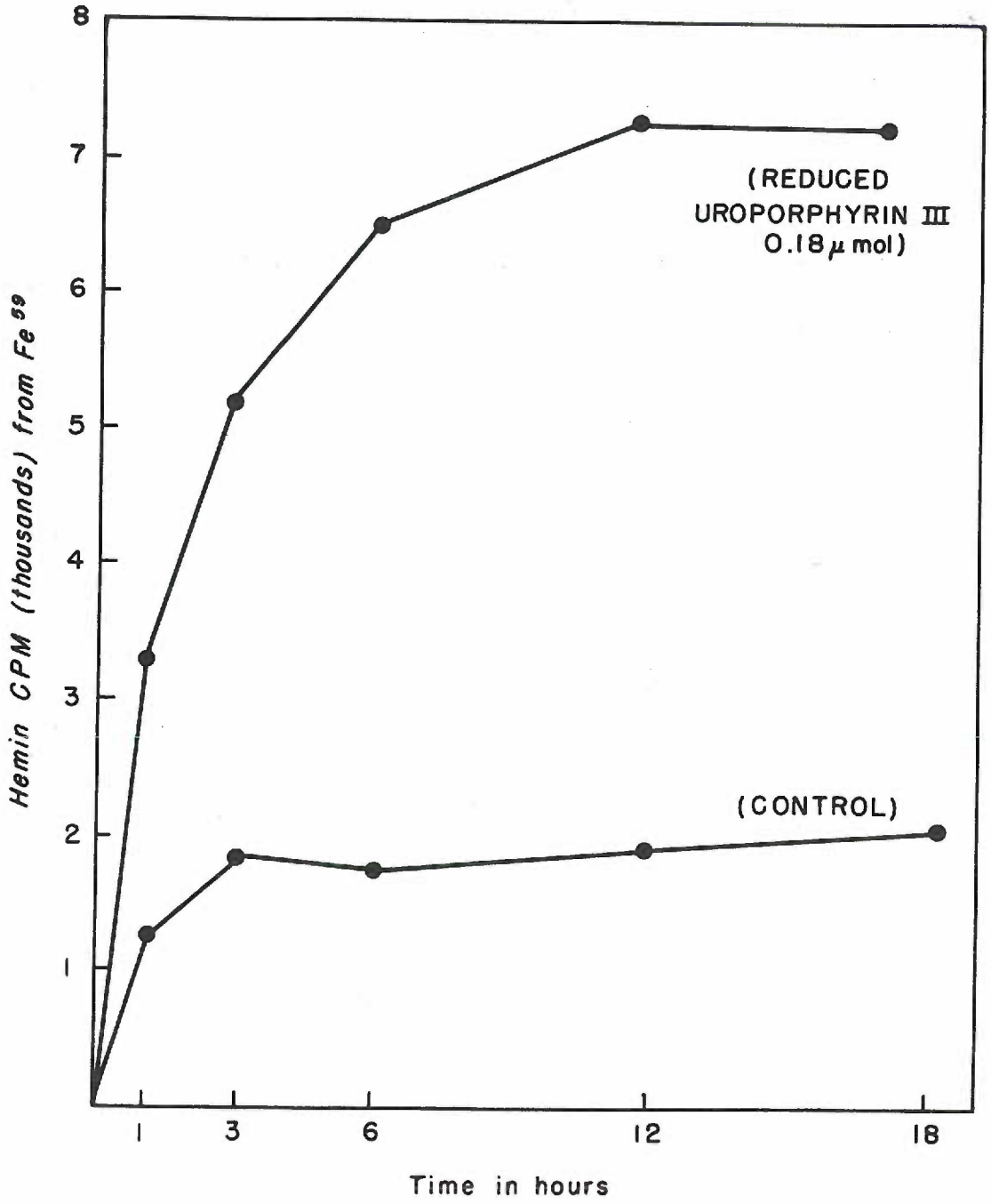
Incubation for various time intervals of the reduced uroporphyrin III with the lysed cell system and $Fe^{59}$, again produced this increase in the radioactivity of the heme (Fig. 3), indicating increased biosynthesis.

It would not have been surprising to find extremely high concentrations of reduced uroporphyrin III resulting in a lowered uptake of $Fe^{59}$, however, the inhibition occurring

**Figure 4**

**Time factor in Reduced Uroporphyrin III Conversion to Heme in Duck RBC.**

TIME FACTOR IN REDUCED - UROPORPHYRIN III
CONVERSION TO HEME IN DUCK RBC



## TABLE IX

### $Fe^{59}$ Uptake with Added Reduced Uroporphyrin III

### During Heme Biosynthesis

| Reduced Uroporphyrin III (μ M / sample) (max. amt.) | Hemin Activity (CPM) |
|---|---|
| 0 | 1870 |
| 0.1 | 6000 |
| 0.2 | 7100 |
| 0.3 | 18000 |
| 0.4 | 7500 |
| 0.6 | 4530 |
| 0.8 | 1750 |
| 1.0 | 850 |

Incubation: 9 hrs. at 37° C.

Lysed Blood was Homogenized.

with levels somewhat above 0.3 μ Mole was unexpected. This is thought to be due to the presence of an inhibitor, possibly a urobilinoid substance formed during reoxidation of the reduced uroporphyrin (56), or perhaps an impurity accompanying the uroporphyrin III during its isolation (Table IX).

d) Uroporphyrin I: In the reduced form, this porphyrin was inert toward $Fe^{59}$ uptake (Table X).

e) Coproporphyrin III: As seen in Table XI reduced coproporphyrin III can serve as a heme precursor but appears to be less effective than reduced uroporphyrin III.

The Establishment of Reduced Uroporphyrin III as a Direct Heme Intermediate.

In order to determine whether reduced uroporphyrin III is a direct intermediate in heme biosyathesis, an isotope dilution experiment was carried out using glycine-2-$C^{14}$, a known precursor of heme. Dilution of the labeled intermediate produced by condensation of glycine-2-$C^{14}$ and succinic acid by a non-labeled suspected intermediate should result in a diminution of heme activity. The data in Table XII show a 50% reduction in glycine incorporation in the presence of reduced uroporphyrin III. These data are particularly significant in view of the very low levels of reduced uroporphyrin III present. $Fe^{59}$ uptake was followed in

TABLE X

$Fe^{59}$ Uptake with Added Reduced Uroporphyrin I

During Heme Biosynthesis

| Reduced Uroporphyrin I (μ Moles) | Hemin Activity (CPM) |
|---|---|
| 0 | 980 |
| 0.1 | 970 |
| 0.3 | 960 |
| 0.6 | 880 |

Incubation: 9 hrs. at 37° C.

Lysed Blood was Homogenized.

## TABLE XI

### $Fe^{59}$ Uptake with Added Reduced Coproporphyrin III

### During Heme Biosynthesis

| Reduced Coproporphyrin III (μ Moles) (max. amt.) | Hemin Activity (CPM) |
|---|---|
| 0 | 990 |
| 0.1 | 3570 |
| 0.2 | 3300 |
| 0.4 | 2940 |

Incubation: 9 hrs. at 37° C.

Lysed Blood was Homogenized.

## TABLE XII

### Glycine-2-$C^{14}$ Dilution by Reduced Uroporphyrin III During Heme Biosynthesis

| Reduced Uroporphyrin III (μ Mole) (max. amt.) | Hemin Activity (CPM) |
|---|---|
| 0 | 82 |
| 0.15 | 50 |
| 0.22 | 40 |
| 0.29 | 37 |
| **Porphobilinogen (μ Mole)** | |
| 0.88 | 40 |

27 μ Mole (1.3 μc.) glycine-2-$C^{14}$ per sample

The $C^{14}$ activity was determined with an end window counter. The hemin activity values are corrected to infinite thickness.

Incubation: 9 hrs. at 37° C.

duplicate samples, thereby eliminating the possibility of an inhibition. Thus, the reduced uroporphyrin must have diluted the labeled glycine, and is, therefore, in the main pathway of heme biosynthesis. An approximately equimolar amount of porphobilinogen diluted the $C^{14}$ incorporation to the same degree as the reduced uroporphyrin (Table XII). Thus it appears that reduced uroporphyrin III is in the main pathway of heme biosynthesis.

As indicated by the hypothesis of Shemin, reduced uroporphyrin III is perhaps the first tetrapyrrole formed. Rimington also states that the reduced form of the porphyrin is the one immediately formed. His work (57) and that of Case (11) show an increase in uroporphyrin upon boiling or aeration of the porphobilinogen solution in acid. This oxidation involves the loss of hydrogen atoms from the bridge carbons. Depending upon the side chain arrangement of the monopyrrole, there could be a reduced tetrapyrrole structure (octa, tetra, or dicarboxylic) with the bridge carbons in the reduced state. Exposure to air apparently is sufficient to oxidize the reduced compound to uroporphyrin, coproporphyrin or protoporphyrin. Therefore, one might also expect to find uroporphyrin, coproporphyrin and protoporphyrin produced as by-products of the heme pathway. In one experiment the lysed duck cells were incubated with 170 micrograms of reduced uroporphyrin III in the following manner. Set A consisted of two lysed blood samples; one control

sample and one sample with 170 μg. of reduced uroporphyrin III were incubated at room temperature for three hours. Set B was prepared similarly and incubated for nine hours. The results shown in Table XIII indicate that reduced uroporphyrin III had been converted to coproporphyrin and protoporphyrin and essentially to the same degree after three or nine hours of incubation.

In order to quantitate these conversions additional information was required. The amount of heme synthesized could be calculated from radioactivity measurements. The amount of uroporphyrin formed on reoxidation of the reduced uroporphyrin III and likewise the amount of coproporphyrin and protoporphyrin could be quantitated. The reduction process forms urobilinoid substances, therefore, the quantitation of the actual amount of reduced uroporphyrin available for conversion could be determined by taking a volume of the buffered reduced uroporphyrin solution equal to that added to the lysed blood samples, and adding it to a volume of water equal to the lysed blood sample volume, and incubating it under the same conditions as the blood samples. One could assume that the amount of reoxidized uroporphyrin formed would then be a measure of the amount of reduced uroporphyrin available. The results of this experiment are tabulated in table XIV. Of the 160 μg available for reduction, 36 μg remained for reoxidation and conversion to succeeding intermediates. The total free porphyrin recovered was found to be

## TABLE XIII

### Conversion of Reduced Uroporphyrin III to Coproporphyrin and Protoporphyrin

| | Coproporphyrin µg / 15 ml. * | | Protoporphyrin µg / 15 ml. * | |
|---|---|---|---|---|
| substrate | 3 hrs. | 9 hrs. | 3 hrs. | 9 hrs. |
| None | 0.0075 | 0.0075 | 2.03 | 1.65 |
| Reduced uroporphyrin III (0.2 µ Mole) | 0.35 | 0.29 | 7.20 | 7.20 |

* 15 ml. of lysed blood per sample was used in this experiment.

Incubation: 37° C.

Lysed Blood was Homogenized.

## TABLE XIV

### A Balance Study of the Conversion of Reduced Uroporphyrin III to Uroporphyrin, Coproporphyrin, Protoporphyrin and Heme

| | | |
|---|---|---|
| Uroporphyrin III before reduction ( max. amt.) | | 160 µg |
| Reoxidized Uroporphyrin III | | 36 µg |
| Amount of Reduced Uroporphyrin assumed to be available for conversion | | 36 µg |
| Porphyrin recovery: | Uroporphyrin | 2.28 µg |
| | Coproporphyrin | 1.43 |
| | Protoporphyrin | 7.80 |
| | Total | 11.51 µg |

Calculation of the amount of reduced uroporphyrin III converted to heme.

Microcuries of $Fe^{59}$ added to lysed blood sample 1.5 µc.

Amount of iron added as ferrous citrate / sample 11 µg.

Activity added per sample:

$3.7 \times 10^4$ disentegrations / sec / µc

$\times \frac{4.5 \text{ (µc added to total blood volume of 90 ml.)}}{16.65} \times 10^4$ D / second

$\times \frac{60}{99.9 \times 10^5}$ D / min / 90 ml. = $33.3 \times 10^5$ D / min / 30 ml. or D / sample

Counter efficiency equals 50 per cent

2 Kcnts / min / sample = D / min.

Amount of heme in 30 ml. of blood (9 ml. of cells) is 125 mg.

The activity of the heme is measured from 2 mg. aliquots, therefore, multiply the D / 2 mg. sample by 62.5 for total heme activity.

TABLE XIV ( cont'd)

Heme activity:

| | cnts / min | D/ min | x 62.5 | total D / min. |
|---|---|---|---|---|
| control | 575 | 1150 | 7187 | 72000 |
| sample | 4193 | 8386 | 534125 | 524000 |

Activity due to reduced uroporphyrin III 525,000
- 72,000
452,000 D / min.

Per cent labeled $Fe^{59}$ incorporated into heme

$$\frac{0.45 \times 10^6}{3.33 \times 10^6} \times 100 = 13.6\ \%$$

11 μg of Fe added / sample

$$\frac{11}{56} = 0.2\ \mu \text{ Moles / sample}$$

μ Moles of iron bound in heme

$$0.2 \times 0.136 = 0.027\ \mu \text{ Moles}$$

Therefore, the amount of reduced uroporphyrin utilized = 0.027 μ Moles

830 μg equals 1 μ Mole of reduced uroporphyrin

.027 μ Moles equals 16.3 μg

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| | |
|---|---|
| Total erythrocyte porphyrin | 11.51 μg |
| Reduced uroporphyrin converted to heme | 22.41 μg |
| Total conversion | 33.92 μg |

Per cent conversion:

$$\frac{33.9}{36.0} = 94\ \%$$

11.51 μg. The amount of reduced uroporphyrin converted to heme was calculated to be 16.3 μg. The combined values equal 27.8 μg for a total recovery of 77 per cent.

Identification of the porphyrins formed from reduced uroporphyrin III was obtained by analysis of the absorption peaks of the extracted uroporphyrin, coproporphyrin, and protoporphyrin and were 628 mμ, 402, 408 respectively. These values are similar to the peaks found for known pure compounds (Table XV).

In view of information presented by Goldberg (44), protoporphyrin was reinvestigated as an iron acceptor. The results showed that in the presence of phosphate ion, $Fe^{59}$ is incorporated into protoporphyrin to some extent (Table XVI). However, the uptake is much less than that with reduced uroporphyrin III.

## TABLE XV

### Absorption Analysis of Porphyrins Formed from Conversion of Reduced Uroporphyrin III

| | Maximum | |
|---|---|---|
| | Literature | Observed |
| Uroporphyrin | 628 | 626-628 |
| Coproporphyrin | 402 | 400-404 |
| Protoporphyrin | 408 | 408-410 |

## TABLE XVI

### $Fe^{59}$ Uptake During Heme Biosynthesis with Added Protoporphyrin in the Presence of Phosphate Ion

| Protoporphyrin µg | Hemin Activity (CPM) |
|---|---|
| 0 | 150 |
| 139 | 420 |
| 278 | 440 |
| 556 | 770 |

Incubation: 3 hrs. at 37° C.

Lysed Blood was Homogenized.

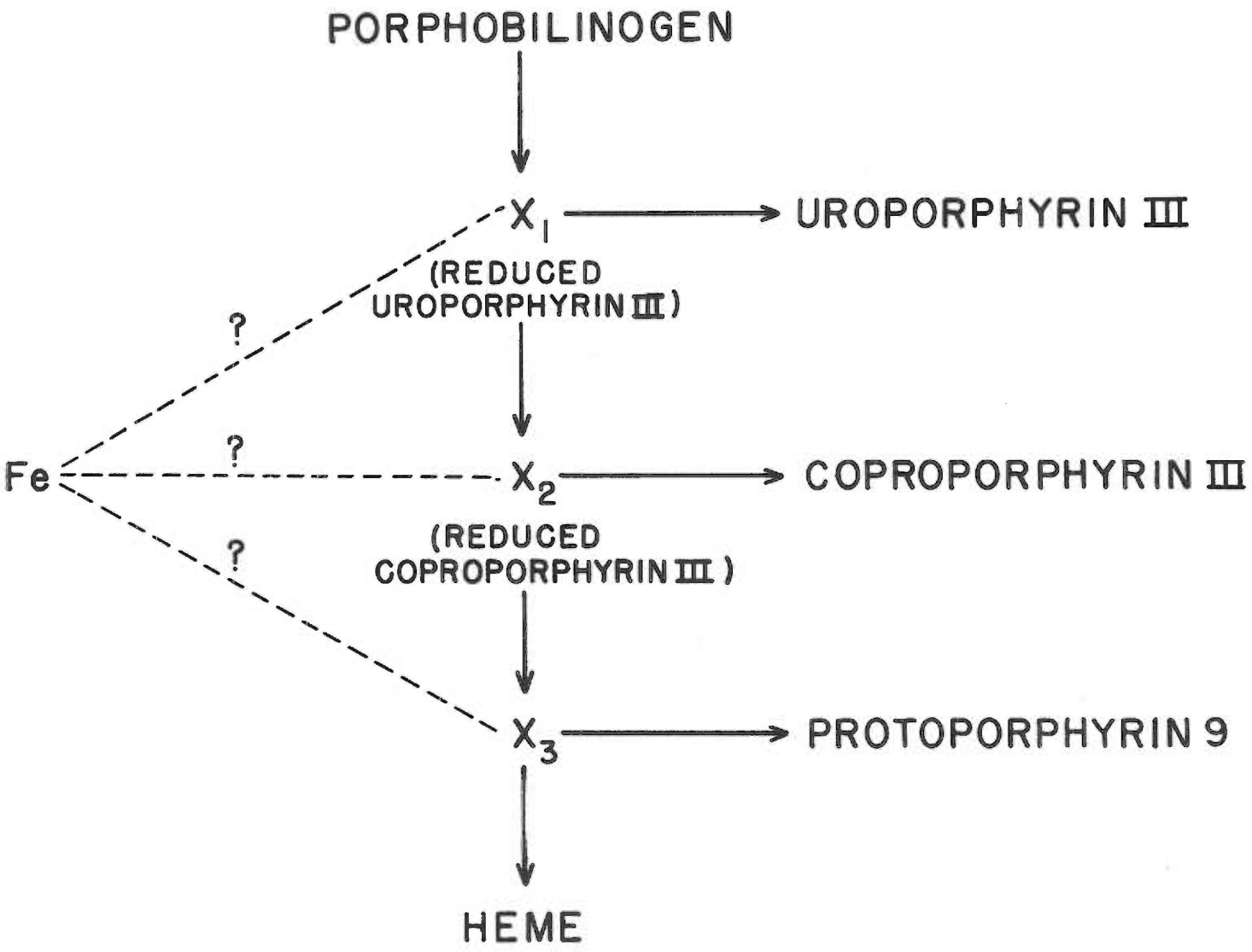
# DISCUSSION

Material presented in the foregoing pages certainly suggests that uroporphyrin and coproporphyrin, in the reduced state, are in the direct pathway of heme biosynthesis. The concept that the naturally occurring tetrapyrroles usually seen, namely, uroporphyrin, coproporphyrin, hematoporphyrin, and protoporphyrin are not in the main biosynthetic pathway has been partly borne out; i. e., the only active compounds (uroporphyrin III and coproporphyrin III) must be in the reduced state, thus partly confirming the theoretical considerations of Shemin.

The dilution of glycine-2-$C^{14}$ incorporation into heme by reduced uroporphyrin III suggests that reduced uroporphyrin III is a direct heme intermediate. Furthermore, since the isotope dilution by porphobilinogen is similar to that seen to occur with reduced uroporphyrin III, the two heme precursors must lie close together in the pathway. Thus the porphyrinogen $X_1$ in Figure 5 is probably reduced uroporphyrin III. Additional confirmatory evidence for the natural occurrence of reduced tetrapyrroles has recently been noted. Bogorad (58) found a colorless compound produced enzymically from porphobilinogen, which yielded uroporphyrin I, slowly by autoxidation in air, and rapidly by the action of a plant enzyme. Furthermore, this colorless compound, when

Figure 5

A suggested pathway of heme biosynthesis.


PORPHOBILINOGEN
X1
UROPORPHYRIN III
(REDUCED
UROPORPHYRIN III)
?
Fe
?
X2
COPROPORPHYRIN III
(REDUCED
COPROPORPHYRIN III)
?
X3
PROTOPORPHYRIN 9
HEME

incubated with a Chlorella cell preparation, yielded, not uroporphyrin, but coproporphyrin (isomer type not determined) as the main product. This suggests that the compound served as a substrate for the enzymes mediating decarboxylation of the protoporphyrin precursors. Granick (59) has separated three protein fractions from the soluble portion of chicken erythrocytes by paper electrophoresis. Fraction 1 contained an enzyme which catalyzed the condensation of δ-aminolevulinic acid to porphobilinogen. Fraction 2 was capable of converting porphobilinogen to a colorless compound which on autoxidation was identified as uroporphyrin III. Fraction 3 together with fraction 2 converted porphobilinogen to colorless compound which on autoxidation was shown to be coproporphyrin III. Of great interest was the finding that the oxidized uroporphyrin produced by the action of fraction 2 on porphobilinogen could not be acted upon by the decarboxylating enzyme in fraction 3. This finding may be interpreted as strongly suggesting that decarboxylation of the porphyrins can occur only in the reduced state.

Reduced coproporphyrin III was also shown to accept $Fe^{59}$ during heme biosynthesis, but apparently to a lesser degree than the reduced uroporphyrin III. Additional experiments are indicated here to clarify the position of this intermediate. In view of the observations of Bogorad and Granick, it is very likely

to appear in the heme biosynthetic scheme at the position $X_2$ in Figure 5.

In the normal human erythrocyte, the presence of coproporphyrin III and protoporphyrin 9 has been established. The presence of uroporphyrin III under normal conditions may be assumed in order further to establish the existence of the scheme. It has been shown that reduced uroporphyrin III serves as a direct heme precursor, and therefore the oxidized derivative is off the main pathway. Rapid conversion of the reduced compound to coproporphyrin may account for lack of evidence for its existence. The normal erythrocyte levels of free coproporphyrin and protoporphyrin indirectly suggest the possibility that only extremely small and as yet undetected amounts of free uroporphyrin are actually present under normal circumstances, as seen below:

Free Erythrocyte Porphyrins (54)

µg %

| Uroporphyrin | Coproporphyrin | Protoporphyrin |
|---|---|---|
| ? | 0.3 | 13.0 |

The pool size of the free erythrocyte porphyrins would indicate that the equilibrium is shifted to the right thus accounting for the greater amount of protoporphyrin normally found, and the observed absence of uroporphyrin.

Early experiments using lysed duck erythrocytes with added glycine and succinic acid showed very marked synthesis of free protoporphyrin, and also of coproporphyrin. This finding has been confirmed by Orten (60). While free uroporphyrin has not been found in these studies, it has been identified in the erythrocytes of patients with erythropoietic porphyria and Cooley's anemia (61, 32).

Hematoporphyrin, though structurally related, cannot be assigned a position in the direct pathway of heme biosynthesis on the basis of experimental evidence, although its formation in some instances is difficult to explain otherwise.

The role of protoporphyrin, long assumed to be the acceptor of iron and therefore a direct heme intermediate, remains questionable. As mentioned earlier protoporphyrin was the most difficult of all the porphyrins to maintain in physiologic solution. After reduction, this problem was even greater. Adding to the uncertain status of this compound is the confirmation of Goldberg's work showing uptake of $Fe^{59}$. The only apparent difference, and thus the important condition in the method of Goldberg, is the presence of ortho phosphate ion, which apparently facilitates $Fe^{59}$ incorporation. Preliminary studies, therefore, reveal that phosphate ion is necessary, but the finding has not been pursued further. This observation is not too surprising if one considers that it

may be reduced protoporphyrin which is biochemically active and therefore the added protoporphyrin would need to be reduced for activity. Relative to this suggestion, examples can be cited to show the necessity for phosphate in certain oxidation-reduction enzymes of a type which may very likely be active here. A direct correlation of enzyme activity with phosphate concentration was shown by Kearney (62) studying the requirement of succinic dehydrogenase for inorganic phosphate. The reduction of cytochrome c by xanthine oxidase has also been shown to be dependent on the presence of inorganic phosphate (63).

It should be recalled here that Dresel concluded, from experiments cited earlier, that protoporphyrin did not dilute glycine-2-$C^{14}$ significantly and therefore did not fulfill the requirement of a direct heme intermediate. This finding by no means denies the possibility that a small amount of protoporphyrin has proceeded to heme, since there actually was a small but detectable amount of glycine dilution. Because the glycine and protoporphyrin are relatively far removed from one another in the biosynthetic pathway, a trace of protoporphyrin may show virtually no dilution of glycine-2-$C^{14}$ and yet show significant uptake of $Fe^{59}$. The important point is that in this reaction sequence, in which the intermediates are proximate to one another, the $Fe^{59}$ uptake should have far exceeded the amount observed, if the protoporphyrin were the principal binding

agent. The results show, however, that less than 0.3 μ Mole of reduced uroporphyrin III was capable of producing heme with an $Fe^{59}$ activity greater than nine times the control level, while that from 1 μ Mole of protoporphyrin was only 3 to 4 times the control, even in the presence of phosphate. This comparison of $Fe^{59}$ uptake indicates that reduced uroporphyrin III has about nine times the activity of protoporphyrin as a heme precursor. Furthermore, the data suggest that iron may be incorporated at some intermediate phase rather than as the final step in heme formation ($X_3$ in Fig. 5).

The inactivity of reduced uroporphyrin I with regard to $Fe^{59}$ uptake indicates additional work which may possibly establish the relationship of the type I and III isomers. It is possible that the reduced uroporphyrin I may proceed in a manner similar to that suggested for uroporphyrin III, and the lack of enzymes prevent the derivatives from proceeding beyond coproporphyrin, thus accounting for the observed lack of protoporphyrin I in nature.

Thus, experiments have been described which were designed to elucidate the site of iron incorporation. While this locus remains in a yet undetermined state, a new intermediate in heme biosynthesis, perhaps the first tetrapyrrole, has been established and the long unknown position of uroporphyrin clarified.

## SUMMARY

1. The incorporation of iron into naturally occurring tetrapyrroles has been investigated in a lysed duck erythrocyte preparation.
2. Uroporphyrin, coproporphyrin, hematoporphyrin, and protoporphyrin did not show any significant uptake of $Fe^{59}$ when incubated with the lysed duck erythrocytes.
3. Reduction of these porphyrins was carried out with sodium amalgam. These reduced derivatives were then investigated as heme precursors using $Fe^{59}$.
4. Reduced uroporphyrin III stimulated heme biosynthesis when added in amounts up to 0.3 μ Mole. Greater concentrations caused inhibition, probably due to the presence of urobilinoid substances formed by the reduction process.
5. Reduced uroporphyrin I, reduced hematoporphyrin 9, and reduced protoporphyrin 9 showed no increased labeling of hemin with $Fe^{59}$.
6. Reduced coproporphyrin III stimulated heme biosynthesis but to a lesser degree than reduced uroporphyrin III.
7. Glycine-2-$C^{14}$ incorporation into heme was markedly diminished in the presence of reduced uroporphyrin III. The isotope dilution was similar to that produced by porphobilinogen, suggesting that reduced uroporphyrin III is in the main pathway of heme biosynthesis.

8. Reduced uroporphyrin III was found to be converted to uroporphyrin, coproporphyrin, and protoporphyrin, as well as to heme.
9. It is suggested that iron may be incorporated at some intermediate phase other than protoporphyrin.
10. Oxidized porphyrins appear to be side products of heme biosynthesis.
11. Phosphate ion appears to be necessary for heme biosynthesis from protoporphyrin. However, the synthesis is less than that from reduced uroporphyrin III.

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AN ABSTRACT OF THE THESIS OF

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## ABSTRACT

The utilization of tetrapyrroles in heme biosynthesis has been studied in lysed duck erythrocytes with radioactive iron ($Fe^{59}$) incorporation into heme used as a measure of heme synthesis. Protoporphyrin, long assumed to be the acceptor of iron in heme synthesis, could not be demonstrated as the principal agent for iron incorporation under either aerobic or anerobic conditions. There was no correlation between the resultant heme radioactivity and protoporphyrin disappearance, similarly, the other naturally occurring tetrapyrroles, uroporphyrin, coproporphyrin, and hematoporphyrin yielded only negative results.

Since the common tetrapyrroles could not be established as heme precursors by virtue of iron acceptance, attention was turned to their reduced derivatives. Reduction of the porphyrins, accomplished with sodium amalgam, results in the addition of hydrogen atoms to the four methene bridge carbon atoms and two

of the pyrrole nitrogen atoms. Rapid reoxidation of the colorless non-fluorescent reduced derivatives was retarded by the addition of ascorbic acid.

The reduced derivatives of protoporphyrin, hematoporphyrin, and uroporphyrin I were shown to be incapable of incorporating $Fe^{59}$.

Reduced uroporphyrin III, however, showed marked uptake of $Fe^{59}$ at levels up to 0.3 $\mu$ Mole. Above this level inhibition was noted, which is considered to be due to the presence of urobilinoid substances formed during the reduction process.

Reduced coproporphyrin III was also noted to increase heme synthesis but to a lesser degree than reduced uroporphyrin III.

Reduced uroporphyrin III was shown to be a direct intermediate in heme biosynthesis by an isotope dilution experiment using glycine-2-$C^{14}$, a known precursor of heme. The results showed a 50% reduction of glycine incorporation into heme in the presence of reduced uroporphyrin III. An approximately equimolar amount of porphobilinogen diluted the $C^{14}$ incorporation to the same degree as the reduced uroporphyrin III.

Reduced uroporphyrin III was found to be converted to free uroporphyrin, coproporphyrin, and protoporphyrin as well as to heme, supporting the theoretical considerations that reduced

uroporphyrin III is the first tetrapyrrole formed.

Protoporphyrin was recently reported to bind iron during heme biosynthesis, results which are in contrast to those obtained in this laboratory. Experimental confirmation was accomplished with the finding that phosphate ion is necessary for this incorporation. Nevertheless, reduced uroporphyrin III was found to be nine times more active than protoporphyrin in heme synthesis.