SEDORMID-INDUCED PORPHYRIA IN THE RAT

By

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A THESIS

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PREFACE

Dr. Robert Aldrich, acting on the hypothesis that the porphyrin-containing enzymes are of prime importance in the pathogenesis of various disease states, established in the summer of 1952 with the cooperation of the Department of Biochemistry a laboratory devoted to the study of porphyrin metabolism. Believing also that porphyria represents a metabolic defect in protoporphyrin synthesis and is therefore a key biochemical disease, Dr. Aldrich set as one of the first goals of the laboratory the development of a satisfactory porphyric experimental animal. It is felt that this goal has been achieved by producing an experimental porphyria in rats fed Sedormid. These studies form the basis of this report.

I wish to thank Dr. R. S. West for his constant guidance and Dr. Robert Aldrich for his help and tutelage in these studies. I am also very grateful to Richard Reve, who helped characterize the excreted porphyrins.

INTRODUCTION

Biochemical interest in the porphyrins centers around the compound protoporphyrin, which combines with iron to form the important prosthetic group heme. Heme acts in different capacities in respiration in the compounds hemoglobin, myoglobin, cytochrome c, cytochrome oxidase, catalase, and peroxidase.(1) Protoporphyrin is probably also used to synthesize chlorophyll.(2)

There is growing evidence that uroporphyrin and coproporphyrin are intermediates or byproducts of protoporphyrin synthesis. Uroporphyrin and coproporphyrin are contained in tissues and excreta in only trace amounts normally, but are present in relatively huge, and chemically variable, amounts in porphyria. Porphyria then, is perhaps a key biochemical disease, useful in the study of the intermediary metabolism of the heme compounds.

In addition to its fundamental relation to porphyrin metabolism, porphyria deserves attention because elucidation of the basic metabolic error could benefit humans afflicted with the disease.

Evidence from the literature which links uroporphyrin and coproporphyrin with protoporphyrin synthesis will be presented here, as well as a history of experimental porphyria and the background of the experimental approach.

Structural formulae of important compounds to be discussed in this paper are presented in figure 1.

A. Evidence that Uroporphyrin and Coproporphyrin are linked with Protoporphyrin Synthesis.

Hoppe-Seyler(3) in 1871 found that sulfuric acid acted upon blood to form a purple pigment which he called "hematoporphyrin".

MacMunn[4] in 1880 showed the presence of porphyrin in normal urine, while Salkowski[5] and subsequent workers described porphyrin excretion in certain diseases. These investigators all considered these porphyrins to be hematoporphyrin because their spectroscopes could not distinguish the artificial hematoporphyrin from the naturally-occurring porphyrins.[6] Early investigators therefore concluded that urinary porphyrins resulted from hemoglobin breakdown.[7]

Although Hans Fischer was able to show that hematoporphyrin differs from urinary porphyrins, and, in fact, does not occur in nature, the idea that free porphyrins result from hemoglobin breakdown still persists.[8] Experimental evidence, however, does not support this theory, but instead indicates that free porphyrins in nature are precursors or byproducts of protoporphyrin synthesis. Since porphyria becomes a key biochemical disease if uroporphyrin and coproporphyrin are involved in protoporphyrin synthesis, some evidence on this question will be presented.

Dobriner et al.[9] found that when hemopoiesis is increased in humans and experimental animals coproporphyrin excretion paralleled reticulocyte formation rather than bile pigment formation.

More convincing, however, was Dobriner's discovery that the urinary coproporphyrin under conditions of increased hemopoiesis is of the type I configuration. Protoporphyrin IX of heme is of the type III configuration. It is universally agreed that conversion of type III to type I porphyrin, which would involve breaking the porphyrin ring and reassembling the pyrroles, does not occur in nature.[10]

The constant ratio of coproporphyrin I formation to protoporphyrin of hemoglobin formation caused Dobriner and Rhoads,[11] to postulate that coproporphyrin I is formed as a byproduct of hemoglobin synthesis.

Figure I

STRUCTURAL FORMULAE OF PORPHYRINS AND SEDORMID*

Legend: Ac = $-CH_2-COOH$

Pr = $-CH_2-CH_2-COOH$

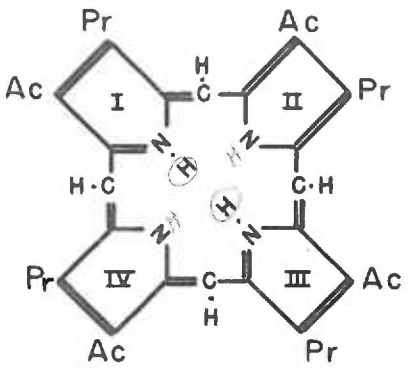
M = $-CH_3$

V = $-CH=CH_2$

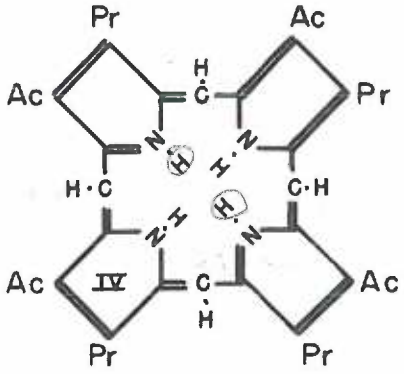
Note: Type I and III isomers differ by their order of side chains in ring IV. See uroporphyrin.

Reference: Dobriner and Rhoads[10]

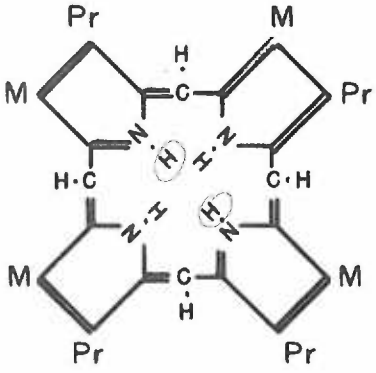
*Allylisopropylacetylcarbamide



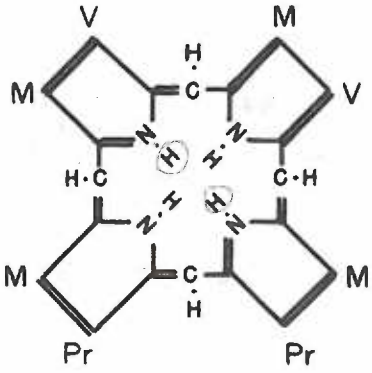
Uroporphyrin I



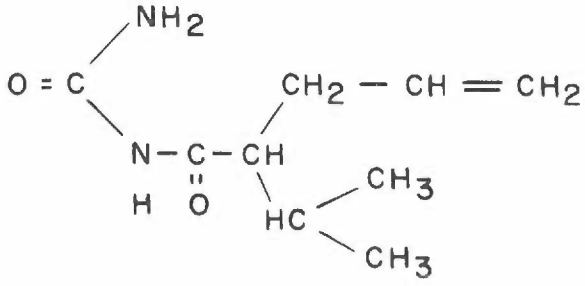
Uroporphyrin III



Coproporphyrin III



Protoporphyrin IX



Sedormid

Even in lead poisoning, where the large excesses of urinary coproporphyrin have been shown to be of type III, evidence favors a derangement of hemoglobin synthesis rather than degradation. Francis and Litzner[12] found that porphyrin excretion parallels the rate of hemopoiesis, rather than the rate of hemoglobin breakdown. Kark and Meiklejohn[13] found that if hemoglobin is injected intravenously in patients with lead poisoning, bilirubin excretion is increased, while porphyrin excretion is not. Bleeding, according to de Langen and Grotepass,[14] markedly increases porphyrinuria in lead poisoning.

After the discovery by Schwartz of the presence of free coproporphyrin in the erythrocyte,[15] and his identification of this compound as the type III isomer, considerable evidence has accumulated which implicates coproporphyrin III in hemoglobin synthesis. A high correlation of erythrocyte coproporphyrin and reticulocyte levels in various anemias suggests that the erythrocyte coproporphyrin is a sensitive index of the rate of hemoglobin synthesis and is a normal precursor of hemoglobin protoporphyrin.[16]

Granick[17] has used irradiation of algae to produce mutants lacking in key enzymes in order to increase the concentration of chlorophyll intermediates. Bogorad and Granick[18] irradiated a protoporphyrin-producing mutant and isolated another mutant which excreted porphyrins having properties of uroporphyrin and coproporphyrin. They felt that this last irradiation had destroyed enzymes necessary for protoporphyrin synthesis, allowing accumulation of intermediates.

Salomon et al.[19] incubated C-14 labeled uroporphyrin III with rabbit bone marrow and recovered half of the activity in protoporphyrin.

The brilliant isotope studies on the biosynthesis of protoporphyrin

IX offer another clue as to the role of uroporphyrin. Contributions of several groups since 1945 showed glycine and acetate to be specific precursors of protoporphyrin. These contributions culminated in the work of Shemin and Wittenberg[20] in 1951 in which they completely degraded the labeled protoporphyrin molecule and were able to assign relative activities to each of the component carbon atoms. They concluded that all four pyrrole rings are manufactured from a common pyrrole, which has the structure of the pyrrole of uroporphyrin. They were unable, however, to say whether alteration of this pyrrole in protoporphyrin formation takes place before or after linkage of pyrroles occurs. If decarboxylation and dehydrogenation occur after the pyrroles are linked together into a porphyrin, uroporphyrin III is a precursor of protoporphyrin IX.

Evidence that coproporphyrin is not a precursor of protoporphyrin was offered in recent work by Rees and Starr.[21] Working with a yeast strain with no respiratory activity or cytochrome absorption bands, these workers found that catalase and cytochrome C were produced if protoporphyrin was added to minimal medium. However, the addition of coproporphyrin III produced no catalase activity.

B. History of the Experimental Animal

There are two broad groups of porphyrinopathies, or diseases in which porphyrin excretion is markedly increased.[22] One group is the porphyrinurias, in which the increase is of the ether-soluble porphyrin, mainly coproporphyrin. A notable example of this class of disorders is found in lead poisoning, in which coproporphyrinuria is extreme. The other group consists of the porphyrias, in which both ether-soluble porphyrin and ether-insoluble porphyrin or uroporphyrin,

occur in large amounts in the urine. Since the present report deals with an experimental porphyria, a review of this subject will be presented.

Stokvis[23] appears to be the first investigator to produce an experimental porphyria. He had observed in 1889 a port-wine colored urinefrom an ill woman who died after being treated with sulfonal over a long period of time. Spectroscopically the urine pigment resembled hematoporphyrin. Several subsequent cases were reported, so Stokvis in 1895 administered sulfonal to rabbits and dogs to see whether "hematoporphyria" could be produced. He spectroscopically detected "hematoporphyrin" in the urine of these animals after a few days of sulfonal administration. He also observed that the porphyrin was not preformed in the urine, but appeared after exposure to air or a small amount of acid. Subsequent studies showed that the urine probably contained uroporphyrin, an unknown compound in Stokvis' time.

Fischer and Duesberg[24] found uroporphyrin in the urine of rabbits with sulfonal poisoning. Waldenström and Wendt[25] found increased urinary coproporphyrin III in sulfonal poisoned rabbits, with inconstant small amounts of ether-insoluble porphyrin. Doberiner[26] isolated a uroporphyrin from a known human case of sulfonal poisoning. Other investigators have reported negative results with sulfonal in rabbits.[27]

Turner[28] summed up the status of sulfonal-induced porphyria by saying, "Some rabbits chronically poisoned with sulfonal excrete uroporphyrin, and these may be profitably studied; yet such animals lead a precarious existence and the induction of porphyria in them is by no means easy or constant."

Schwartz, Keprios, and Schmid[29] recently reported an experimental porphyria in rabbits. These authors found that lead or phenylhydrazine injections increased urinary uroporphyrin levels, but highest levels were achieved by combining phenylhydrazine, lead and exposure to light. The urinary uroporphyrin was of type I configuration. Uroporphyrin was present in large amounts in bone marrow but only trace amounts in liver. These findings are similar to those observed in congenital porphyria in humans. Porphobilinogen was, however, present in the urine of the animals, a finding considered pathognomonic of human acute porphyria.[22]

Schmid and Schwartz[27] published a report in December, 1952, of a very different type of porphyria produced in rabbits by Sedormid* ingestion. Extremely high urinary uroporphyrin levels were recorded, chiefly of type III configuration. Large amounts of urinary porphobilinogen were present. Tissue analysis showed large amounts of uroporphyrin and porphobilinogen in the liver, while porphyrin content of bone marrow and erythrocytes was within normal limits. These chemical findings are similar to those seen in acute human porphyria.

Another approach to artificially-produced porphyria has been provided by Bogorad and Granick[19] in their recent work with irradiated algae. A mutant of Chlorella vulgaris, which ordinarily produces chlorophyll, has been isolated which manufactures porphyrins with carboxyl groups corresponding to coproporphyrin and uroporphyrin.

Aside from these attempts to artificially induce porphyria, there are a number of situations in which uroporphyrin occurs in appreciable amounts in nature, which represent potential or actual

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*Allylisopropylacetylcarbamide, manufactured by Hoffmann-La Roche, Inc., Nutley, New Jersey.

experimental animals.

Turner in 1937[28] discovered that the fox squirrel, Sciurus niger, enjoys a physiologic porphyria. The urine and bones of these animals are colored red with uroporphyrin I, and the bone marrow contains large amounts of uroporphyrin I. This type of porphyria corresponds to the congenital, or "erythropoietic"[30] porphyria of humans. Though Turner said that the fox squirrel is "common...and becomes quite tame," Schwartz et al.[29] state that "preliminary studies with several of these animals indicated that the practical difficulties involved in handling them limits their usefulness."

Congenital porphyria was discovered in a South African herd of cattle in 1936[31] and an animal was subjected to chemical examination by Rimington.[32] The results closely resembled the findings of Fischer in the famous case Petry, a congenital porphyric, even to the point of photosensitization of the light-colored portions of the hide.

Clare and Stephens[33] have reported congenital porphyria in the pig.

Some other possible experimental animals, generally somewhat inaccessible and not easily handled, are: Pteria vulgaris, a mollusc found in tropical waters in whose shell Fischer and Hauser[34] found uroporphyrin I; Asio flammeus, a bird of the sub-arctic, whose bones according to Derrien and Turchini,[35] contain uroporphyrins and African birds of the Turacus genus, whose wing feathers contain a uroporphyrin I copper complex.[36]

Filmantscher[37] has found that the porphyrin deposited in mammalian fetal bones is uroporphyrin.

Of course, humans afflicted with the several types of porphyria

have contributed a major portion of the present knowledge of porphyrin metabolism, and have stimulated much porphyrin research. They present, however, all the limitations inherent in the use of humans as experimental animals, plus the disadvantages of rarity and of being rather extremely ill.

C. Significance of the Experimental Animal and Selection of the Experimental Approach.

In 1937 Turner[28] wrote: "The intermediary pyrrole metabolism must be studied to determine if possible by what steps protoporphyrin, coproporphyrin and uroporphyrin are formed. Finally we must determine what factors make for the normally great excess of type III porphyrin over type I, and under what conditions this relationship is disturbed..... One of the greatest of (difficulties) has been the (lack of a satisfactory experimental animal)." These goals are still largely unmet.

The advent of isotopic tracer techniques has provided tools, which, coupled with the proper experimental animal and well-designed experiments, should make possible the elucidation of the biologic interrelationships of the porphyrins. The evidence presented earlier that uroporphyrin and coproporphyrin are involved in protoporphyrin synthesis strengthens the theory that porphyria represents a metabolic error in protoporphyrin synthesis or utilization. Thus the experimental porphyric animal should prove useful in elucidation of some of the steps in the important biosynthetic chain between the fundamental pyrrole postulated by Shemin and Rittenberg[21] and formation of protoporphyrin IX.

In addition, definition of the metabolic errors in the porphyrias could benefit humans afflicted with these diseases.

Since in this laboratory attention is directed toward the role of porphyrin-containing enzymes in various disease states, an experimental porphyria is primarily intended to test the hypothesis that the signs and symptoms of porphyria represent an intracellular lack of heme-containing respiratory compounds, notably cytochrome c and catalase.

Because the known functionally-important porphyrin-containing compounds are of type III configuration, a type of experimental porphyria was sought which disrupts the synthesis of type III porphyrin within the cell. Since in congenital human porphyria it is the type I porphyrins which are excreted, the relationship to protoporphyrin IX synthesis is not clear. However, in adult, or "hepatic" porphyria,[32] the type III porphyrins are excreted abnormally. It is quite possible that these abnormal amounts of uroporphyrin III and coproporphyrin III represent blocks of enzymatic reactions in the synthesis of protoporphyrin IX.

The rat was selected as the experimental animal because of the vast amount of biochemical information available on the rat, and because rat liver has proved to be an ideal medium for enzyme studies.

Possible methods of producing a porphyria of the "adult" type were limited to the use of several sedative drugs: sulfonal, which had produced equivocal results in animals in other investigators' hands; trional, which had reportedly caused uroporphyrin excretion in humans[38]; barbiturates, which had been reported as precipitating acute porphyria[39] but which failed to increase rabbit urinary porphyrins in the hands of Laubender and Monden[40]; and Sedormid, which in a communication from Dr. Rudi Schmid[41] in the summer of 1952 was cited as causing the excretion of large amounts of uroporphyrin in rabbit urine. Schmid's work had been prompted by the suggestion by Dannberg[42] in 1932 that

prolonged Sedormid ingestion had caused acute porphyria in one of his patients. This last, most hopeful, approach was decided upon: administration of Sedormid to the rat.

Oral administration of Sedormid to the rat has caused a marked change in urinary porphyrin excretion, and has produced a syndrome which chemically and clinically simulates adult human porphyria. These studies constitute the present report.

METHODS

A. Work Done With The Animals

1. Animals Albino Sprague-Dawley rats from the Biochemistry colony were used throughout the experiments. Rats 1 to 4 were 400 gram males; rats 38 and 39 were 60 gram weanling females. All other rats were 200 ± 20 gram females when experiments were begun. These rats were raised on a ration of Purina Laboratory Chow and were fed the same ration during experiments. They had unlimited access to food and water during experiments, though at times they became too weak to eat or drink.

2. Urine Collection Three metabolic cages similar to those described by Guest[43] were constructed. These cages separated fairly efficiently the urine and feces and food, allowing 0-1 grams of food and 0-3 fecal pellets to fall on the glass wool in the funnel during a 24-hour urine collection.

3. Sedormid Suspension Since Sedormid is virtually insoluble in water, saline, and other innocuous solvents, it had to be administered as a suspension. A satisfactory suspending agent for stomach-tubing proved to be propylene glycol. The Sedormid powder was suspended and partially dissolved in propylene glycol by grinding in a mortar or mixing in a Waring Blendor. The usual concentration was 50 mg Sedormid per ml of suspension.

For parenteral injection a satisfactory suspension was made by mixing isotonic glycerol, 0.1% Tween 80, and 20 mg per ml of Sedormid.*

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*I am indebted to Dr. Ellen Talman for devising and preparing this solution.

4. Stomach-tubing To insure an accurate oral dosage, Sedormid was administered by stomach tube. The suspension was given in single daily doses of 200 to 400 mg of Sedormid per kg per day.

5. Parenteral Injections Intraperitoneal and subcutaneous (skin of back) injections of the parenteral Sedormid suspension were made using sterile technic, in single daily doses of 200-400 mg Sedormid per kg per day.

6. Routine of Urine Collections Before placing animals in the metabolism cages, urine was stripped from the bladder and the daily Sedormid injection given. Time of bladder stripping was noted as the start of the urine collection period.

Animals were seen approximately 24 hours after being placed in the metabolism cages. They were removed from the cages after manually stripping bladder urine into the cages. Time of stripping was noted as the end of the urine collection period.

The following were recorded for each animal: (1) strength, as shown by gait of locomotion, (2) alertness, (3) weight, (4) number of fecal pellets excreted, (5) amount and color of urine, (6) amount of food and water taken, (7) presence or absence of rales, nasal secretions, diarrhea, and urine-staining of the ventral abdominal wall, (8) general appearance.

Cages were rinsed with about 30 ml of water, from a wash bottle, which drained into the collecting funnel. Urine plus wash water was transferred to 125 ml Erlenmeyer flasks, and the collecting funnel and beaker were replaced with clean glassware and a new glass wool plug. Urines were then refrigerated prior to determining porphyrin levels.

B. Routine Chemical Determinations

The urine samples, which included wash water, were made up to 100 ml with distilled water in a graduated cylinder. Each sample was then divided, 50 ml being placed in a 125 ml Erlenmeyer flask for determination of "boiled" uroporphyrin (UB). The remaining 50 ml were used for the determination of "unboiled" uroporphyrin (U), coproporphyrin (C), and the qualitative test for porphobilinogen (P).

1. Coproporphyrin Determination Coproporphyrin was determined by the "5-cc" method of Schwartz et al.[44]. In this procedure a 5 ml aliquot of urine is placed in a 250 ml separatory funnel, and 15 ml of distilled water and 5 ml of buffered acetic acid (four volumes glacial acetic acid and one volume of saturated aqueous sodium acetate) are added. This solution is then shaken with 80 ml of ethyl acetate. Coproporphyrin passes into the ethyl acetate phase. The aqueous phase is saved for uroporphyrin determination. The ethyl acetate is then washed twice with 25 ml portions of 1 per cent sodium acetate or until no fluorescence is observed in the aqueous washes under a Woods light*. The ethyl acetate is then shaken with 25 ml of 0.005 per cent iodine, prepared fresh each day from a 1 per cent solution of iodine in ethyl alcohol by dilution with water. This procedure is designed to convert any coproporphyrin precursors to coproporphyrin. Small amounts of ethyl alcohol are used to "break" the emulsion formed at this point. After discarding the aqueous phase, the coproporphyrin is extracted with four 4 ml portions of 1.5 N HCl. The HCl extract

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*Model BL-2, Black Light Products, Chicago, Illinois.

is then made up to 25.0 ml with 1.5 N HCl, shaken, and poured into a fluorimeter tube.

2. "Unboiled" Uroporphyrin Determination The first aqueous phase from the coproporphyrin determination was combined with the first 1 per cent sodium acetate wash, and any subsequent washes that contained visible fluorescence. This fraction contained the uroporphyrin from the 5 ml aliquot of urine. This fraction was made 1.5 N with the appropriate volume of 7.5 N HCl, and about 25 ml were poured into a fluorimeter tube.

3. "Boiled" Uroporphyrin Determination The 50 ml portion of urine to be boiled was adjusted to pH 5.0 using a Beckman pH meter equipped with large electrodes. A boiling stone was added, and the samples were boiled on a hot plate for twenty minutes. After cooling, the samples were diluted to 50.0 ml with distilled water, and a 5 ml aliquot was treated as described above to isolate the uroporphyrin. The ethyl acetate phase, containing coproporphyrin, was discarded.

For porphyrin quantitation, use was made of the fact that porphyrins fluoresce red when struck by light in the near-ultraviolet range. The Calectron fluorimeter, capable of measuring amounts of coproporphyrin as small as 10^{-9} g, was used for this purpose. This instrument has two primary filters, Corning 5543, to isolate the 405 millimicron emitting band, and a secondary filter, Corning 2424, to isolate the emitted red band. A linear relationship exists between fluorescence intensity and porphyrin concentration below 100 micrograms coproporphyrin per 100 ml. Unknown concentrations of porphyrin were measured against a 5 microgram coproporphyrin per 100 ml standard in 1.5 N HCl, using

a blank of 1.5 N HCl. Thus all porphyrin concentrations reported herein are in terms of coproporphyrin fluorescence.

4. Porphobilinogen Test A qualitative test for porphobilinogen was performed on each urine specimen, using the method of Watson and Schwartz[45]. In this test a 5 ml aliquot of urine is added to 5 ml of Ehrlich's reagent (0.7 gram p-dimethylamidobenzaldehyde, 100 ml water, 150 ml of conc. HCl), and, after shaking, the solution is allowed to stand one minute. Then 10 ml of saturated sodium acetate is added with shaking, and the solution is again allowed to stand for one minute. If color develops, 10 ml of chloroform are added with shaking. The test is interpreted as positive if more than a very slight pink color appears in the aqueous phase. An arbitrary grading scale of 0 to ++++ was adopted. Lack of any pink color was 0, + was a very slight pink color, and ++++ was a deep violet.

C. Characterization of Excreted Porphyrins

Further procedures, employing selected urine samples, were carried out in an attempt to identify the excreted porphyrins.

1. Isolation of Porphyrins The Sveinsson technic[46] was used to remove porphyrins from the urine specimens. This method consists in adding calcium chloride and sodium hydroxide to centrifuged urine, which results in a precipitate of calcium phosphates and hydroxide on which porphyrins are quantitatively adsorbed. The centrifuged precipitate was then dissolved in 7.5 N HCl.

2. Formation of Porphyrin Methyl Esters Since purification of porphyrins by chromatography is most conveniently accomplished using the methyl esters, esterification was carried out using a

modification of the method of Schwartz et al.[47]. Porphyrins dissolved in 1.5 N HCl were left standing overnight in the dark with ten volumes of esterifying mixture. The esterifying mixture usually consists of a 20:1 methanol:sulfuric acid solution. Then 25 ml of chloroform were added followed by distilled water until no more chloroform phase appeared. Esterification was judged complete when all fluorescence passed into the chloroform phase. The chloroform phase was washed five times with distilled water and once with 7 per cent sodium chloride. The esters were then taken to dryness for storage or purification.

Experiments using zinc chloride or trichloroacetic acid as catalysts instead of sulfuric acid gave satisfactory esterification, but difficulties in separating catalyst from ester made these compounds less satisfactory than sulfuric acid.

3. Chromatography of Porphyrin Methyl Esters When purification of uroporphyrin methyl ester or its separation from coproporphyrin ester was desired, chromatography was carried out according to the method of Schwartz et al.[47]. In this method a 15 mm chromatography tube is tightly packed with Merck calcium carbonate as the adsorbing agent. Dry porphyrin methyl ester is dissolved in 3 drops of chloroform and diluted with 2 ml of benzene. This solution is placed on the column and developed with mixtures of benzene and chloroform varying in proportions from 15 parts benzene per part chloroform. The higher concentration of chloroform speeds the rate of development. Gentle water suction is used. Impurities remain on the top of the column, uroporphyrin ester is in a band just below the top, and coproporphyrin is further down the column, separated as a discrete band. The calcium carbonate is extruded,

and the uroporphyrin and coproporphyrin zones are cut out under visualization with the ultraviolet light. The porphyrin esters are eluted from the calcium carbonate with chloroform. When further purification is desired the process is repeated.

When only coproporphyrin isomer is desired as a purified product, as for example in isomer ratio determinations, petroleum ether instead of benzene is used with chloroform to develop the coproporphyrin band. Since petroleum ether precipitates uroporphyrin methyl ester, this procedure prevents the possibility of uroporphyrin traveling down the column and contaminating the coproporphyrin.

4. Determination of Coproporphyrin Isomer Ratio The ratio of coproporphyrin III to coproporphyrin I in the isomer preparations was determined using a modification of the fluorescence quenching method of Schwartz et al.[47]. An aliquot of chloroform containing 5-10 micrograms of total purified coproporphyrin ester is dried in a fluorimeter tube. The ester is dissolved in 5 ml of redistilled acetone and 10 ml of Sørensen phosphate buffer (pH 6.0). A fluorimeter reading is taken after mixing. The tube is then frozen for six minutes in a beaker of acetone mixed with dry ice. The tube is allowed to stand at room temperature for sixteen hours, and a second fluorimeter reading is made. The ratio of the first to second fluorimeter readings gives the isomer ratio when applied to curves previously constructed from known mixtures of pure coproporphyrin I and III standards. This method takes advantage of the fact that fluorescence of coproporphyrin I is lost or "quenched" after freezing, whereas that of coproporphyrin III is unaltered.

5. Decarboxylation of Uroporphyrin To determine the isomer configuration of the enriched uroporphyrin, partial decarboxylation of purified uroporphyrin to coproporphyrin was carried out, and the isomer ratio of this coproporphyrin was determined as outlined above. For the decarboxylation the recent modification by Edmundson and Schwartz[48] of the standard method of Fischer and Hilger[49] was used. This difficult procedure, carried out by Richard Nevd, was not modified, and will not be described in detail here. The method utilizes the fact that mild decarboxylation (0.3 N HCl, 2-25 mm Hg pressure, 150° C. for three hours) proceeds only as far as coproporphyrin.

6. Preparation of Uroporphyrin Methyl Ester Crystals Uroporphyrin methyl ester was crystallized out of chloroform by adding 5 ml of redistilled methanol to a Kahn tube containing 0.5 ml of porphyrin-containing chloroform. The tube was shaken, a cotton plug was inserted, and the tube was refrigerated. Flocculent red-brown uroporphyrin crystals appeared after a few hours.

Recrystallization was performed by pouring the mother liquor and crystals over a cotton plug in a small funnel, discarding the filtrate, removing the crystals by repeated chloroform washings, boiling the chloroform down to 0.5 ml and refrigerating again with methanol as described above.

Examination of crystal morphology was carried out with an ordinary microscope and light source.

The melting point of these uroporphyrin methyl ester crystals was determined with a #12-143 Fisher-Jones melting point apparatus. Several melting point determinations were unsuccessful because of sintering.

7. Determination of Absorption Spectra Absorption spectra of the various porphyrins were determined using the Beckman DU spectrophotometer. Solvents were either 1.5 N HCl or chloroform, and the porphyrins were either in the free state or esterified.

8. Separation of Coproporphyrin and Protoporphyrin Since the ethyl acetate phase of the "5-ee" method extracts both coproporphyrin and protoporphyrin[50], and since the rat excretes large amounts of protoporphyrin in his feces[51], due probably to the Harderian gland[50], it was thought desirable to determine whether or not a substantial amount of protoporphyrin was contained in the "copro" fraction. Protoporphyrin has not been reported as a constituent of the urine of any species in any measurable amounts.

A method of separating these two porphyrins was used which utilizes the fact that coproporphyrin can be extracted from ether with 0.1 N HCl, while protoporphyrin requires 3 N HCl. The "copro" in 1.5 N HCl from the "5-ee" method was buffered to pH 5 and extracted with an equal volume of ether. Under these conditions both coproporphyrin and protoporphyrin move into ether[50]. This ether solution was washed twice with 20 ml portions of 0.5 per cent sodium acetate. Coproporphyrin was extracted with five 4 ml portions of 0.3 N HCl. Any protoporphyrin remaining in the ether was then extracted with 3 N HCl.

D. Tissue Studies

1. Rat 32 This animal which had been "porphyric" for one week was examined grossly under the ultraviolet light. The animal received 2 mg of nembutal intraperitoneally, and 5 ml of blood were removed with a heparinized syringe by cardiac puncture. The viscera were examined on both the outside surface and cut section

for red fluorescence under the ultraviolet light. A description of the red fluorescence seen will be presented in "Results".

Some of the organs were assayed for porphyrin content. Heart, kidney, small gut, Harderian gland, brain, spleen, and liver were weighed, homogenised in the Waring Blender with distilled water, made 5 per cent with respect to trichloracetic acid, and centrifuged. These extracts were then treated in the same way as urine specimens for determination of coproporphyrin and "boiled" and "unboiled" uroporphyrin.

Red blood cells were assayed for porphyrin in the following manner: 1.4 ml of packed erythrocytes were treated with buffered acetic acid until the debris was colorless. Then the debris was extracted several times with 1.5 N HCl until fluorescence no longer appeared in the extract. The buffered acetic acid extracts and 1.5 N HCl extracts were combined, neutralised to red-grey to congo red paper, and extracted three times with 2 volumes of ether. Under these conditions uroporphyrin remains in the aqueous phase while protoporphyrin and coproporphyrin are extracted by the ether.(50) The ether solution was then washed three times with 1 per cent sodium acetate, and these washes were added to the original aqueous phase to combine all the uroporphyrin. Coproporphyrin was extracted from ether with 0.1 N HCl and protoporphyrin with 3 N HCl. The porphyrins were quantitated fluorimetrically.

A supernatant of trichloracetic acid-treated plasma was tested for porphobilinogen.

The fluorescent face secretions were washed from the animals with distilled water, the porphyrin was isolated by the Sveinsson technic, and the absorption spectrum determined in 1.5 N HCl

against a blank of 1.5 N HCl.

2. Examination of Organs in Ultraviolet Light After the discovery of bizarre red fluorescence, presumably due to porphyrin, in various parts of the carcass of rat 32, dissections were performed on other rats in a further study of these phenomena.

Rat 33, a 200 gram Sedormid-fed female which had been in trunk porphyria for six days, was sacrificed. The viscera, central nervous system, Harderian glands, and portions of the skeleton and peripheral nervous system were dissected and viewed externally and on cut section in the dark under a Woods light. Results of this examination will be presented later. Rat 42, a normal 200 gram Sprague-Dawley female, was similarly examined. Rat 44, a normal Long-Evans female obtained from Dr. Donald Walker, was also sacrificed.

Since a difference in tissue fluorescence between normal and porphyric rats seemed to exist, nine animals, some porphyric and some non-porphyric, were killed with gas on March 23, 1953, for the purpose of examining the kidneys. Histories and results of examination of these animals will be given later.

3. Fluorescence of Purina Chow After observing that the contents of the rat stomach and gut fluoresced red, examination was made of the Purina chow pellets which made up the rat diet. Grossly the pellets fluoresced a dull orange-brown, and on close inspection small (less than 1 mm) jewel-like particles were seen which fluoresced bright red.

An attempt was made to isolate the red-fluorescing material. Purina chow was mixed with water in a Waring Blendor and centrifuged. The red fluorescence was seen to reside in the top layer of sediment in a non-miscible, light green oil. This oil was poured

off and extracted with ethyl acetate buffered with acetic acid. All of the fluorescence went into the ethyl acetate phase. This fluorescence could not be extracted with 1.5 N, 3 N, 7 N or concentrated HCl.

RESULTS

A. Urinary Porphyrin and Porphobilinogen Excretion

1. Normal Rats Table I presents data on normal urinary porphyrins determined on 13 200-gram Sprague-Dawley female rats. In 59 determinations the range of coproporphyrin was 3.2 to 25.8 micrograms per 24 hours with a mean of 12.2. "Unboiled" uroporphyrin ranged from 0.5 to 13.0 micrograms per 24 hours with a mean of 5.6. "Boiled" uroporphyrin values varied between 1.6 and 13.9 micrograms per 24 hours with a mean of 5.4. In any given urine specimen "boiled" and "unboiled" values were essentially equal, indicating that no appreciable uroporphyrin precursor is present in normal rat urine. All porphobilinogen tests performed on these urine samples were negative. Control values for two weanling rats, 38 and 39, fell within the ranges given in Table I for 200 gram females.

2. Effect of Oral Sodomid in Propylene Glycol Table II presents urinary porphyrin excretion values after oral administration of Sodomid suspended in propylene glycol. A total of 22 rats were so treated. In 20 of these same abnormality of porphyrin excretion was produced, with values well above the normal range. The two rats who failed to show increased porphyrin values were rat 6 and rat 18, both of whom died from complications of stomach-tubing after only three days of drug administration. Rats 1, 2, 3, 4, 11, 12, 13, 14, 15, 16, 20, 32 and 33 reached levels sufficiently high to be considered practical sources of uroporphyrin and coproporphyrin.

Coproporphyrin was consistently the first constituent to rise, usually being elevated after the third day of drug dosages. Levels of

Table I

NORMAL RAT URINE PORPHYRIN
(micrograms per 24 hr read as coproporphyrin)

Days	Rat #5			Rat #6			Rat #7		
	C	U	U(B)	C	U	U(B)	C	U	U(B)
1	11	3.3	2.5	14	3.2	1.6	7	0.5	1.2
2	12.8	6.0	-	11.8	4.0	-	7.5	1.2	-
3	11.5	4.5	2.9	16	5.7	3.3	7.5	2.8	2.9
4	10	4.9	-	20	6.6	-	9.5	4.2	-
5	11.6	3.8	-	12.6	4.4	-	9.4	3.3	-
6	14.5	4.0	-	13.5	11.4	-	9.3	3.0	-
7	11.5	4.5	-	12	6.8	-	10	3.5	-
8	12	6.0	7.0	13	9.6	8.4	11.5	3.6	3.9
9	16	6.3	-	14	11.7	-	9.5	3.6	-

C = coproporphyrin
U = uroporphyrin - unboiled
U(B) = uroporphyrin - boiled (isolated by Sveinsson technic)

Note: at no time did any control rat have a positive porphobilinogen test. All rats in Table I were 200 gram female Sprague-Dawleys.

Table I, cont'd

Days	Rat #15			Rat #16			Rat #17			Rat #18			Rat #19			Rat #20		
	C	U	U(B)	C	U	U(B)	C	U	U(B)	C	U	U(B)	C	U	U(B)	C	U	U(B)
1	3.2	5.4	5.5	5.3	4.0	3.6	9.0	5.7	4.7	13.9	5.1	5.6	13.9	5.1	5.6	8.5	6.3	5.6
3	12.2	6.3	6.5	7.1	3.3	3.3	8.2	4.6	4.9	16.2	4.5	4.7	14.1	5.8	5.8	10.6	6.9	6.9
5	16.6	7.2	6.8	5.7	3.1	3.1	7.4	5.3	5.3	17.9	4.6	4.6	24.5	6.6	7.4	22.2	8.3	9.4
7	25.8	13.0	13.9	9.4	4.0	4.2	15.6	5.2	4.9	17.4	6.1	6.1	17.5	4.5	4.1	12.5	7.5	6.4

Days	Rat #21			Rat #22			Rat #23			Rat #24		
	C	U	U(B)	C	U	U(B)	C	U	U(B)	C	U	U(B)
2	9.0	6.3	6.1	10.6	7.8	6.4	17.6	9.0	9.0	4.2	8.2	8.4
4	7.0	6.4	6.4	11.5	9.4	9.7	22	7.5	9.5	5.5	6.3	6.8

	C	U	U(B)
Av'g of all	12.2	5.6	5.4
Range of all	3.2-25.8	0.5-13.0	1.6-13.9

Note: U(B) on this page was isolated by ethyl acetate in solubility instead of Svolinsson technic.

TABLE II

RAT URINARY PORPHYRINS AFTER ORAL SEDORMID ADMINISTRATION

24-HOUR EXCRETION LEVELS

(EXPRESSED AS MICROGRAMS PER 24 HRS. READ AS COPROPORPHYRIN)

Days Drug Admn.	Rat 1 400 g. ♂		Rat 2 400 g. ♂		Rat 3 400 g. ♂		Rat 4 400 g. ♂		Rat 5 200 g. ♀				Rat 6 200 g. ♀			
	C	U	C	U	C	U	C	U	C	U	U(B)	P	C	U	U(B)	P
1	21		18						15.4	2.3	3.0	+	13	5.5	5.0	0
2					87.5	4.2	85.	4.7	17.7	8.0	8.4	0	22.5	4.8	5.8	0
3	84	6.3	71	7.0					71.	6.8	9.4	+	22	7.6	6.4	0
4					144.	42.	178.	15.1	65.9	9.8	12.8	—	DIED - PROBABLY OF PNEUMONIA			
5		6.3	95.	10.6	96	24			10.2	12.4	9.2	0				
6					DIED OF DRUG EFFECT		222	30.5	47.4	7.4	6.8	0				
7	81	7.3	102.	11.8			DIED OF ASPHYXIA		30.4	6.4	3.9	0				
8					* STRONGLY POSITIVE PORPHOBILINOGEN TEST DISCOVERED				41.6	16.9	10.2	0				
9	70.2	—	97	13.3												
10									13.3	6.9	3.1	0				
11	128	15.6	143.	15.8					DIED OF DRUG EFFECT AND PERI. PNEUMONIA							
13	103	13.5	125	15.8												
15	DIED OF ASPHYXIA		179	26.7												
17			80	11.7												
19			104	17.8												
20			20	6 ? DIED OF ASPHYXIA												
Area Result Drug	200 Mg/Kg		280 Mg/Kg		400 Mg/Kg		400 Mg/Kg		200 Mg/Kg				300 Mg/Kg			

Legend:

C = coproporphyrin

U = uroporphyrin, "unboiled"

U(B) = uroporphyrin, "boiled"

P = porphobilinogen test

(0 & + are negative, #, ##, ### are positive)

TABLE II, (CONT'D)

Days Drug Admin.	Rat 9 200 g. ♀				Rat 10 200 g. ♀				Rat 11 200 g. ♀				Rat 12 200 g. ♀				Rat 13 200 g. ♀				Rat 14 200 g. ♀			
	C	U	U(B)	P	C	U	U(B)	P	C	U	U(B)	P	C	U	U(B)	P	C	U	U(B)	P	C	U	U(B)	P
8													68.4	17.5	17.	+++	28.8	8.0	6.0	+	65.9	28.5	22.	+++
9													60.	8.5	15.	+	84.6	13.3	17.5	+++	33.4	9.0	7.5	0
11													119.	23.6	100.	++++	61.4	18.8	21.4	+	60.6	16.3	—	+++
12													106.	16.4	131.	++++	39.2	12.3	11.5	+	61.	9.3	10.7	0
13													146.	24.5	380.	++++	19.5	10.6	9.9	+	91.	11.0	24.4	0
14													142.	64.	1225.	++++	58.	13.9	16.4	0	78.	12.5	16.	0
15													150.	103.	1572.	++++	80.	16.5	19.	+++	92.	14.5	45.	+++
16													136.	116.	1239.	++++	93.	16.7	116.	+++	84.	21.8	105.	+++
18												Sudanmil Stopped →	12.3	109.	571.	++++	DIED OF ADENOMA				95.4	43.1	55.	++
19													36.9	20.2	21.5	+++					138.	35.2	175.	+++
20													12.5	12.3	11.1	0					149.	42.5	191.	+++
21													10.8	13.0	11.0	0					84.	16.3	27.1	+++
22													14.1	11.3	9.0	+++					86.	15.7	27.6	+++
23												Sudanmil Suspension →	57.6	13.0	11.6	+++					100.	21.0	34.5	+
25									98.	42.5	1250.	++++	72.	15.1	25.1	+++					123.	26.	80.5	+++
26	70.	28.	36.8	+++									87.6	11.6	18.6	+++					101.	20.1	73.1	+++
27					67.5	19.5	33.3	+++					81.6	13.6	97.6	+++					134.	23.	106.	+++
28													108.	24.7	125.	+++								
29									176.	108.	349.	+++	108.5	27.7	146.	+++					—	—	1280.	++++
30	EXPERIMENT DISCONTINUED				ANIM. D'C'D.				DIED OF DRUG EFFECT				87	21.8	98.4	+++					ANIMAL TERMINAL (SACRIFICED)			
32													61.4	14.7	48.4	+++								
34													80.5	19.9	95.2	+++								
35													106.	19.4	149.	+++								
													EXPERIMENT D'C'D.											
Amts. Daily Drug	200 mg/kg				200 mg/kg				200 mg/kg				300 mg/kg				300 mg/kg				300 mg/kg			

TABLE II. (CONT'D)

Days Drug Administered	Rat 15 1.00 q. 8				Rat 16 200 q. 8				Rat 17 200 q. 8				Rat 18 200 q. 8				Rat 19 200 q. 8				Rat 20 200 q. 8			
	C	U	U(B)	P	C	U	U(B)	P	C	U	U(B)	P	C	U	U(B)	P	C	U	U(B)	P	C	U	U(B)	P
1	23.8	13.0	13.9	Lost	9.4	4.0	4.2	0	18.6	8.2	4.8	+	17.4	6.1	6.1	+	17.3	4.5	4.1	+	12.5	7.5	6.4	0
3	31.8	9.0	9.2	0	10.2	5.8	4.2	+	14.0	21.	20.5	+	20.6	4.8	6.7	+	32.0	6.9	7.7	0	19.2	18.0	20.8	0
5	34.6	11.5	9.7	0	14.8	5.7	6.3	+	38.7	10.4	11.8	+	Died of Asphyxia				32.5	7.0	6.7	+	51.8	23.8	25.5	+
7	52.4	8.9	10.3	+	28.6	6.6	6.4	+	53.4	4.0	57.6	+++					38.8	7.1	12.0	+	39.1	39.1	30.5	+
9	68.0	9.2	25.2	+	29.2	5.0	8.4	+	38.7	4.8	67.5	+++					34	0.8	0.6	0	30.2	8.5	8.3	+
11	189.	98.5	1254.	+++	81.	7.4	9.4	+	Died of Drug Effect								Death Unexplained				44.4	29.7	92.1	+++
12	51.5	32.7	98.2	+++	66.6	8.8	14.4	+									(Probably Drug Effect)				13.8	48.4	113.8	+
13	154.	278.	1080.	+++	48.2	10.9	11.5	+													Died of Drug Effect			
14	57.9	30.4	30.7	+	108.	11.8	23.5	0																
15	21.8	21.0	20.8	0	98.5	11.3	18.0	+																
16	38.5	20.9	23.2	0	108.	13.1	17.0	+																
17	Died of Drug Effect				118.	12.9	31.8	+																
18					111.	25.2	110.	+++																
19					58.9	11.8	81.	+++																
20					136.	38.8	451.	+++																
21					107.8	51.3	684.	+++																
22					73.2	23.0	9.2	+++																
23					58.1	38.8	28.3	+++																
24					21.8	14.6	15.6	++																
26					7.3	3.3	5.0	+																
27					10.3	4.7	12.6	0																
					Died of Drug Effect																			
Approx. Daily Dose	320 mg/kg				350 mg/kg				300 mg/kg				300 mg/kg				380 mg/kg				350 mg/kg			

TABLE II, (CONT'D)

Days Death Animal	Rat 32 200 g. ♀				Rat 33 200 g. ♀				Rat 38 62. g. ♀ Weanling				Rat 39 57 g. ♀ Weanling			
	C	U	U(B)	P	C	U	U(B)	P	C	U	U(B)	P	C	U	U(B)	P
Control (0)									9.9	4.1	4.1	0	8.7	5.8	5.6	0
1									13.7	7.6	8.2	0	10.5	4.6	4.5	0
2									22.6	4.6	5.7	0	31.8	9.4	13.9	+
3									40.7	5.7	4.4	+	67	34	31.3	++
4									42.7	5.2	82.5	+++	28.2	10.0	15.5	+
5									48.	12.5	19.6	0	61.3	18.3	52.5	+
6									65.9	15.1	47.2	+	34.7	11.1	20.3	0
8									29.6	7.3	22.6	+	36.4	11.2	16.	+
									Experiment Discontinued							
13	92.	34.3	438.	+++					Wt. on 8th day: 74 grams				68 grams			
14	238.	168.	1130	+++												
16					114	99.8	400	++								
	Animals sacrificed for tissue study															
Avg. Daily Dose	300 Mg/Kg				300 Mg/Kg				400 Mg/Kg				400 Mg/Kg			

TABLE III

RAT URINARY PORPHYRINS AFTER PARENTERAL INJECTIONS

Days Drug Admin.	Rat 21 200 G. ♀ C	U	U(B)	P	Rat 22 200 G. ♀ C	U	U(B)	P	Rat 23 200 G. ♀ C	U	U(B)	P	Rat 24 200 G. ♀ C	U	U(B)	P
Control -3	9.0	6.3	6.1	+	10.6	7.8	6.4	0	17.6	9.0	9.0	0	4.2	8.2	8.4	0
Control -1	7.0	6.4	6.4	+	11.5	9.4	9.7	+	22.	7.5	9.5	+	5.5	6.3	6.8	+
0	Intraperitoneal Sedormid* Inj. Begun				Intraperitoneal Control** Inj. Begun				Subcutaneous Sedormid Inj. Begun				Subcutaneous Control Inj. Begun			
3	17.7	5.7	6.3	+	13.7	4.1	4.5	0	18.8	7.6	9.0	+	6.9	6.8	7.2	++
5	10.8	5.6	7.0	+	12.7	4.0	4.4	+	26.8	6.9	9.0	+	8.5	4.7	5.2	+
7	13.3	3.3	4.6	+	15.2	5.4	6.2	+	21.4	6.4	6.3	0	7.5	6.8	6.4	0
9	13.5	5.4	5.4	0	11.6	8.4	8.2	0	25.4	6.8	6.7	0	7.5	6.8	6.3	0
11	12.5	4.7	5.2	0	13.5	5.2	5.0	0	25.2	6.5	7.2	0	8.4	9.1	8.8	0
	Died - Probably of Drug Effect				Experiment D'C'D.				Animal Lost				Experiment D'C'D.			
Avg. Daily Dose	100 Mg/Kg.				1 ml Control Soln.				100 Mg/Kg.				1 ml Control Soln.			

Days Drug Admin.	Subcutaneous Daily 200 Mg/Kg. Sedormid C	U	U(B)	P	Rat & No. Description
36	17.0	10.2	10.6	0	26 - 200 G ♀
36	24.0	9.2	9.2	0	27 - 200 G ♀
36	18.0	14.1	14.7	0	28 - 200 G ♀

Days Drug Admin.	Subcutaneous Daily 400 Mg/Kg. Sedormid C	U	U(B)	P	Rat & No. Description
35	8.5	3.	3.1	0	29 - 200 G ♀
37	20.4	8.0	7.6	0	30 - 200 G ♀
37	21.9	7.0	6.2	0	31 - 200 G ♀

NOTE * Sterile suspension of Sedormid (20 Mg/ml) in isotonic glycerol and 0.1% Tween 80 given in a single daily dose.

** Sterile solution of isotonic glycerol and 0.1% Tween 80 given in a single daily dose

TABLE IV

RAT URINARY PORPHYRINS

PROPYLENE GLYCOL* CONTROLS

DAYS DRUG ADMIN.	RAT T 200 G. ♀				RAT 40 200 G. ♀				RAT 41 200 G ♀			
	C	U	U(B)	P	C	U	U(B)	P	C	U	U(B)	P
1	0.1	0.8	0.9	0								
2	3.2	1.0	0.3	0								
3	3.7	0.8	0.8	0								
4	4.3	4.0	4.6	0								
5	4.1	3.5	3.4	0								
21					11.8	4.1	3.7	0	13.1	6.0	5.7	0
AVG. DAILY DOSE	1.0 ML				1.2 ML				1.2 ML			

* EASTMAN

"boiled" and "unboiled" uroporphyrin remained low and essentially equal for a few days to two weeks, when a sharp increase in both occurred. Simultaneously a positive porphobilinogen test appeared, and the animals became ill. "Boiled" uroporphyrin values, which rose as high as 3230 micrograms per day, exceeded "unboiled" values, indicating the presence of a non-fluorescent uroporphyrin precursor. This precursor, if it were the same as occurs in human acute porphyria, might be porphobilinogen, though this is a disputed point.(52)

Figure II charts a typical chemical and clinical course of an animal receiving oral Sedormid.

3. Effect of Parenteral Sedormid Injections Intraperitoneal Sedormid injections in rat 21 and subcutaneous injections of Sedormid in 7 other rats caused no increase of urinary porphyrins or porphobilinogen out of the normal range, as shown by Table III. The intraperitoneal dosage was held to 100 mg per kg because this dose in rat 21 was regularly followed in 5 to 10 minutes by profound coma with slow, irregular diaphragmatic breathing but without signs of peritoneal irritation. Subcutaneous dosages of 400 mg per kg were estimated to produce lethargy approximately comparable to 200 mg per kg orally. At autopsy of rat 21, the peritoneal surfaces appeared normal, suggesting that Sedormid had been mobilized from the intraperitoneal space.

4. Effect of Oral Propylene Glycol Propylene glycol administered alone by stomach tube failed to cause any increase in urinary porphyrins or porphobilinogen in three rats, as shown in Table IV.

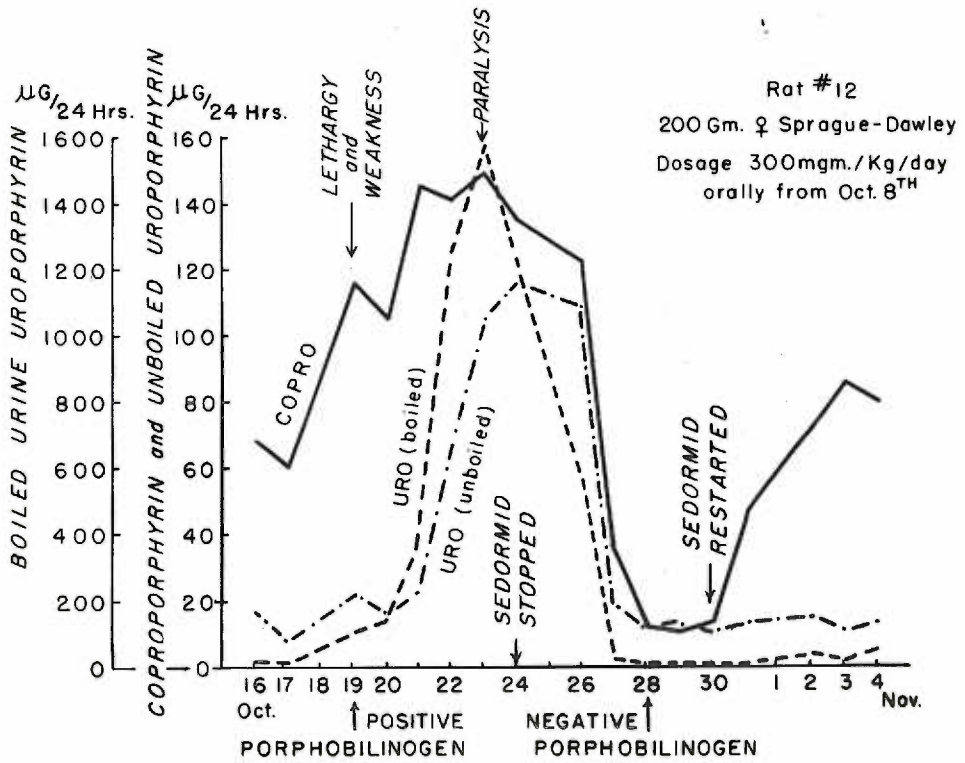
B. Clinical Effects of Sedormid

Normal rats, when first given Sedormid in propylene glycol by

Figure II

TYPICAL CHEMICAL AND CLINICAL COURSE OF AN ANIMAL GIVEN SEDORMID IN PROPYLENE GLYCOL BY STOMACH TUBE

Note: Ordinates are urinary porphyrin values expressed as micrograms of coproporphyrin per 24 hours.


BOILED URINE UROPORPHYRIN
µG/24 Hrs.
1600
1400
1200
1000
800
600
400
200
0
COPROPORPHYRIN and UNBOILED UROPORPHYRIN
µG/24 Hrs.
160
140
120
100
80
60
40
20
0
LETHARGY and WEAKNESS
PARALYSIS
COPRO
URO (boiled)
URO (unboiled)
SEDORMID STOPPED
SEDORMID RESTARTED
Rat #12
200 Gm. ♀ Sprague-Dawley
Dosage 300mgm./Kg/day
orally from Oct. 8TH
16 17 18 19 20 22 24 26 28 30 1 2 3 4
Oct.
Nov.
POSITIVE PORPHOBILINOGEN
NEGATIVE PORPHOBILINOGEN

stomach tube, exhibited signs of acute intoxication-lethargy and weakness of the extremities. These signs began 1-2 hours after intubation and lasted a few hours.

After 1-2 weeks of 300 mg/kg dosages, a number of clinical changes took place coincidentally with a sharp rise in uroporphyrin excretion and appearance of a strongly positive porphobilinogen test. Signs of lethargy and paresis of the extremities (more prominent in the hind limbs) became marked and lasted throughout the 24 hour period between doses. The number of fecal pellets reduced sharply. Urine staining of the ventral abdominal wall appeared, probably because the animals voided while lying unconscious in one position through most of the day. Weight usually dropped during this period, though rat 16 began eating excessively and gained weight.

If dosages were continued, the animals invariably went downhill and died in a comatose state, completely paralyzed, nearly unresponsive, with slow, irregular diaphragmatic respirations. Gross examination of the organs after death was unremarkable except that the stomach was usually packed with 15-25 grams of food.

If Sedormid was stopped at any stage in this downhill course, the animals usually made a dramatic recovery within about 2 days. Lethargy first disappeared, paralysis of the extremities receded, fecal pellet numbers rose to normal or above, while porphyrin excretion levels and the porphobilinogen test reverted to normal (see figure II). Within three days the rats were eating well and had only a slight weakness of the hind limbs, manifested as a rolling gait, to show for their illness.

Other interesting clinical findings seen in some of these animals

were: sticky, purple, red-fluorescent eye secretions; behavior disorders perhaps denoting hunger, such as constant biting at all objects, eating of feces, and cannibalism; various central nervous system signs such as rhythmic bobbing of the head and convulsions involving the forelimbs.

C. Characterization of Excreted Porphyrins

1. Uroporphyrin Methyl Ester Approximately 1.5 mg of purified uroporphyrin methyl ester was prepared from pooled samples of urine from rat 15, collected at the height of drug response. This preparation traveled as a single band on the calcium carbonate chromatography column.

Crystals of this compound recrystallized twice out of methanol melted sharply at 260 to 261° C. (uncorrected). This is near the melting point of uroporphyrin III octamethyl ester, which is said by Rimington[53] to be 255 to 260° C. The Waldenström uroporphyrin methyl ester isolated from cases of human acute porphyria melts at 258 to 260° C.[50] and is perhaps uroporphyrin III[7]. Previous attempts to obtain sharp melting points on uroporphyrin preparations from other "porphyric" rats were unsuccessful because of sintering.

These uroporphyrin methyl ester crystals from rat 15 had under high magnification the appearance of rosettes of long curved hairs, as shown in Figure III.

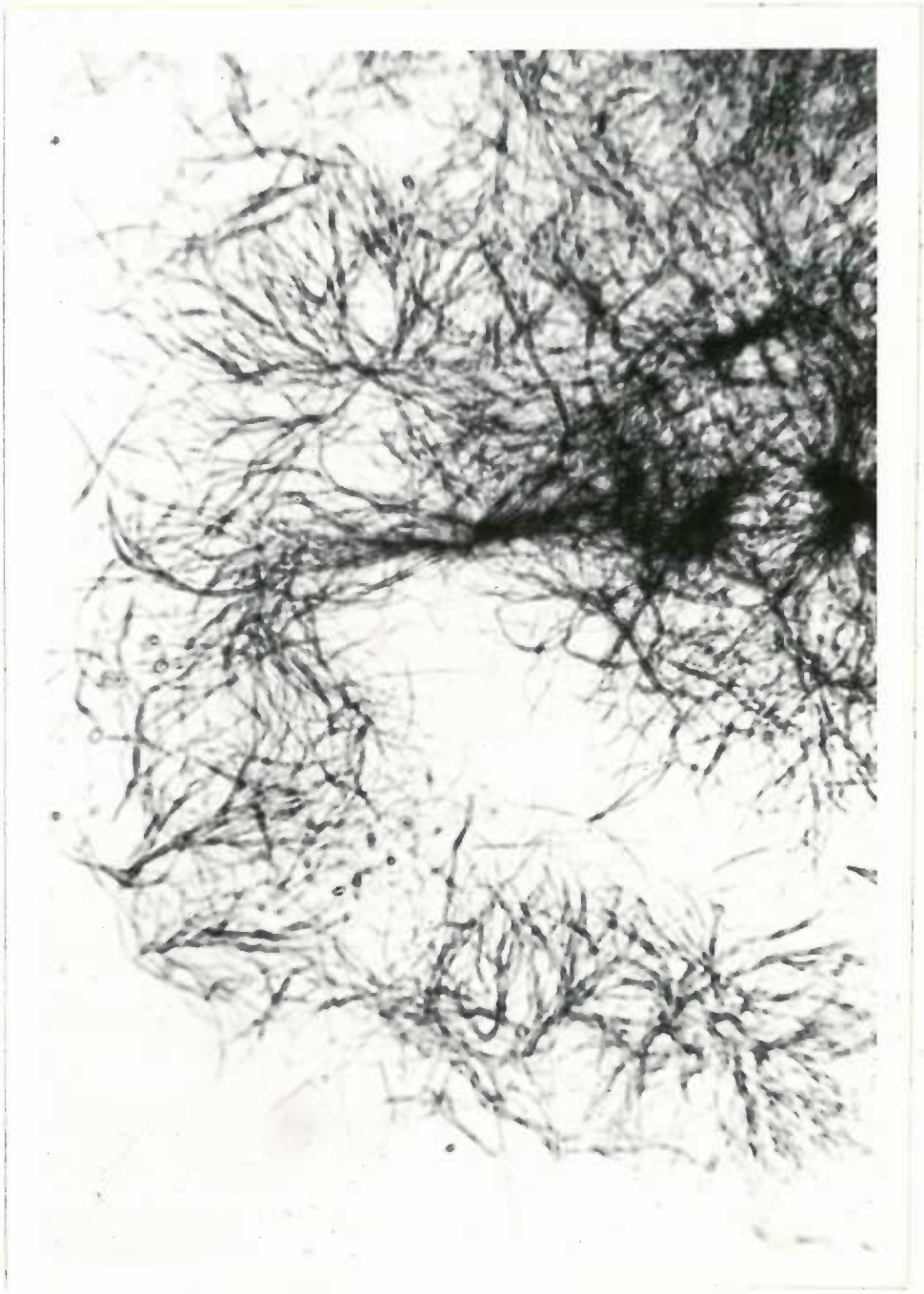
Two 100 microgram samples of this uroporphyrin were successfully in HCl decarboxylated to coproporphyrin by Richard Nevé. He determined the isomer ratio of this coproporphyrin to be greater than 90 per cent type III on both samples. Thus practically all, if not all, of the excreted uroporphyrin must be of the type III configuration.

Figure III

APPEARANCE OF UROPORPHYRIN METHYL ESTER

CRYSTALS FROM URINE OF RAT 15

Note: Magnified 1650 x.



An absorption peak at a wave length of 625 millimicrons was obtained on purified chloroform solution of uroporphyrin methyl ester from urine of rat 12. A known sample of coproporphyrin in chloroform run simultaneously exhibited a maximum at 622 millimicrons. Lemberg[7] lists maxima of 626 and 622.5 for these compounds respectively. Another pooled uroporphyrin sample in 1.5 N HCl from high-uroporphyrin urine of rats 11, 12, 13, and 14 had maxima at 406, 502, 570 to 572, and 625 millimicrons.

2. Coproporphyrin Methyl Ester Coproporphyrin was isolated from urine, isomerated and chromatographed for isomer ratio determination. Three different samples of rat 16 urine were so treated. Type III isomer made up the majority of this coproporphyrin. Percentages of 80, 80 (duplicates), 100, 100 (duplicates), greater than 90, and greater than 95 (duplicates) were obtained on the three urine samples. These procedures were carried out by Richard Nevo[1].

Coproporphyrin isolated in the routine extractions was verified as being coproporphyrin by several absorption spectra determinations. A pooled sample of coproporphyrin in 1.5 N HCl from rats 11, 12, 13, and 14 had maxima at 402, 499, and 568 millimicrons. Rat 14 coproporphyrin in HCl had maxima at 403 and 622 millimicrons; rat 17 coproporphyrin had maxima at 403 and 550. These maxima are typical of known coproporphyrin in the instrument used.

3. Urine Protoporphyrin Levels The urine of rats 1 and 2 on two separate days after high levels of coproporphyrin excretion had been reached were separately examined to assess the amount of protoporphyrin traveling with coproporphyrin, since the "5-cc" method extracts coproporphyrin and protoporphyrin together.[50] These rats had been

on Sedormid for 6 and 8 days respectively. The fluorescence ascribed to protoporphyrin was found to range in these four determinations from 0.6/72 to 2.5/81 of the total daily coproporphyrin output. It was decided that urinary excretion of protoporphyrin under these conditions if it exists at all, is very small. Therefore separation of protoporphyrin and coproporphyrin as a step in the routine chemical determinations was not adopted.

D. Tissue Studies

1. Rat 32 History: 2/12/53 207 gram female started on 300 mg per kg Sedormid in propylene glycol by stomach tube.

2/25 Showed clinical signs of porphyria (wt. 195, +++ weakness, ++ lethargy), C = 92, U = 54, U(B) = 439, P = +++.

2/26 C = 238, U = 162, U(B) = 1130, P = +++.

3/2 (Day sacrificed) Wt. 160, Weakness +++, lethargy +++.

Gross appearance of this animal under the Woods light revealed diffuse red fluorescence about the nose, with brilliant red fluorescence at points of purple secretions seen in ordinary light. There was slight red fluorescence around the urethral meatus and on the urine-stained area of the ventral abdominal wall. Brilliant red fluorescence of the forepaws and dull red fluorescence of the tail were noted.

Examination of the viscera grossly and on cut section revealed a brilliant discrete band of red fluorescence on cut section of the kidney at the position of the cortico-medullary junction; marked red fluorescence of the washed small bowel wall and a dull orange-red

fluorescence of the feces; no fluorescence in the cerebral and cerebellar hemispheres, but striking red fluorescence in the pons, medulla, cranial nerves, and spinal cord; red fluorescence in the sciatic and intercostal nerves; extremely bright red fluorescence in the Harderian gland; red fluorescence in the chondral portion of the ribs; no fluorescence in muscle, eye, bone marrow, bone, liver, and heart. Stomach, adrenals, pancreas, and ovaries were not examined.

Porphyrin analysis on various organs revealed:

Organ	C	U	U(B)
Heart	0	0	0
Kidney	10.3	3.4	0
Small gut	0.4	0	0
Harderian gland	150	2	0
Brain	0	0	0
Spleen	0	0	0
Liver	2.3	2.3	2.1

Note: All concentrations are expressed as micrograms per gram of wet tissue.

The erythrocyte porphyrin levels were:

Uroporphyrin 0

Coproporphyrin 11.6 micrograms per 100 ml RBC

Protoporphyrin 61.8 micrograms per 100 ml RBC

A positive porphobilinogen test was observed on the supernatant of trichloroacetic acid-treated plasma, indicating perhaps that appreciable quantities of porphobilinogen were circulating in the blood of this animal.

A maximum at 409 millimicrons was observed on the face secretion porphyrin in 1.5 N HCl, which identified this material as protoporphyrin.

2. Rat 33 History: 2/12/53 211 gram female started on 300 mg per kg per day Sedormid in propylene glycol by stomach tube.

2/25 Showed clinical signs of porphyria (wt. 172, convulsions, +++ lethargy, +++ weakness)

2/26 C = 114, U = 100, U(S) = 400, P = ++.

3/2 Animal sacrificed. Paralysed, convulsing, unresponsive, with slow diaphragmatic breathing, brownish-purple stains around eyes, paws, and nose.

The findings of red fluorescence described for rat 32 were confirmed in this rat, and additional areas were examined. Slight red fluorescence was seen on the cut ends of trachea cartilages. Lungs fluoresced slightly, more prominently in the whitish, thickened areas which probably represented pneumonia. Very slight red fluorescence was seen around the porta hepatis; none was seen on cut section of the liver. Food and feces and gut wall from stomach to anus fluoresced brilliant orange-red, except in the antral portion of the stomach, where no fluorescence was present in the food or adjacent stomach wall. The feces had some water-soluble fluorescence. No fluorescence was noted in pancreas, adrenals, or spleen.

3. Normal Rats Rat 43, a normal Sprague-Dawley female, and rat 44, a normal Long-Evans female, were dissected and studied under the Woods light. Location and intensity of red fluorescence were discovered to differ only slightly from that described in the "porphyria" rats

32 and 33.

No fluorescence was seen in the kidneys of either rat. Considerably less fluorescence was noted in the feces, none of it water-soluble. No fluorescent secretions were present about the eyes, nose, or forefeet, but a faint pink fluorescence in the muzzle area of the face was apparent. No fluorescence was seen in the sciatic or intercostal nerves or the chondral rib ends.

A more thorough examination of the central nervous system was made on these rats than on previous animals. Red fluorescence was seen in the internal capsule, corpus callosum, and cerebral peduncles. The spinal cord fluoresced on cut section in the periphery. The conclusion reached was that white matter of the central nervous system fluoresced red, while grey matter did not. Cranial nerves I, II, V, VII, and VIII fluoresced red, while III and VI did not.

4. Rat 29 This animal, which had received 400 mg per kg of Sedormid daily for 35 days, was dissected under the Woods light. The appearance of this animal did not differ from the normal rats, except that fluorescent secretions were present around the eyes and external nares.

Several firm white nodules were present in the subcutaneous tissue of the back where injections had been given. This finding was later confirmed in rats 26, 27, 28, 30, and 31, which had received similar subcutaneous injections. Rats 40, 41, 45, and 46, which had received oral preparations, did not present this finding. It was felt that these nodules were the result of the subcutaneous injections, and possibly contained unabilised Sedormid.

5. Kidneys of Other Rats The kidneys of rats 26, 27, 28, 30, and 31, who had received parenteral Sedormid injections for 46 days, showed no

fluorescence. Neither did the kidneys of rats 40 and 41, who had received oral propylene glycol for 22 days. The kidneys of rats 45 and 46, who had been on dosages of 375 mg per kg oral Sodermid for 18 days, showed the same fluorescence seen in the kidneys of rats 32 and 33.

DISCUSSION

A. Urinary Porphyrin and Porphobilinogen Excretion of Normal Rats

The normal values of urinary coproporphyrin reported here agree well with the data of Hoffbauer, given by Schwartz in his 1950 "Porphyrin Metabolism" Lecture Outlines.(50) In Hoffbauer's series 26 adult female rats, with average weight of 201 grams, excreted an average of 11 micrograms per 24 hours, as against 12.2 for the series reported here. Schwartz did not mention the strain of rats investigated. This data was not published, though Schwartz refers to it as "submitted for publication". Rimington and Hemmings(54) quantitated rat coproporphyrin excretion, but did not publish their control data.

No data was found in the literature concerning uroporphyrin excretion of rats. It should be emphasized that the uroporphyrin values reported here on normal rat urine represent only the red fluorescence of the ethyl acetate-insoluble (ether-insoluble) material in that urine. Uroporphyrin from normal rat urine has not been isolated and characterized by its physical properties, and until this is done its presence should be questioned. However, the probability that uroporphyrin is present is considered to be high. Lemberg and Legge(7) state that "Porphyrins are the only substances with red fluorescence in acid solution which occur in feces or urine.".

The interpretation of porphobilinogen tests was difficult in these normal animals, because occasionally a faint pink color not extracted by chloroform would result from the action of Ehrlich's reagent on normal rat urine. These "one-plus" reactions were arbitrarily considered negative. The possibility exists that traces of

porphobilinogen are present in normal rat urine.

The lack of significant discrepancy between "boiled" and "unboiled" uroporphyrin values in normal rat urine (see Table I) indicates that no appreciable precursor is present which will convert to uroporphyrin upon heating.

B. Effect of Oral Sedormid in Propylene Glycol

Oral administration of Sedormid suspended in propylene glycol consistently caused the excretion of large amounts of uroporphyrin, and can therefore be said to have caused an experimental porphyria. This experimental disorder has many features in common with a type of human porphyria.

The urinary findings in these animals of a positive Ehrlich test for porphobilinogen, increased amounts of type III coproporphyrin, and enormous amounts of uroporphyrin largely in the form of precursor are criteria considered diagnostic of acute porphyria in the human adult.(39) Similarly, the signs of lethargy, paralysis of the extremities, and gastrointestinal dysfunction are characteristic of this human disease.

This form of porphyria should be distinguished from the congenital, or "erythropoietic"(34) type, which is chemically and clinically distinct. In congenital porphyria the circulating red cells contain large amounts of uroporphyrin and coproporphyrin, and the excreted porphyrins are largely of the type I configuration.

After this work was started, Schmid and Schwartz(27) published extensive studies on the effect of oral Sedormid on rabbits. The findings reported here on the rat do not differ importantly from those found in the rabbit. These authors presented tissue porphyrin assays which suggested that the liver was the principle site of

involvement.

The consistent increase in urinary coproporphyrin in advance of uroporphyrin seen in these rats is a feature which has been observed in human acute porphyria.(55)

There seems to be a remarkable correlation between uroporphyrin precursor, represented by the discrepancy between "boiled" and "unboiled" values, and porphobilinogen (see Table II). In the past these substances have been considered to be identical, but Watson(52) has presented evidence that the uroporphyrin precursor in acute porphyria is an Ehrlich-negative substance which can be separated from porphobilinogen.

Of the 22 rats treated with oral Sedormid, only 13 developed high enough porphyrin levels to be considered practical sources of uroporphyrin and coproporphyrin. Seven rats (11, 12, 14, 15, 16, 32, and 33) reached very high uroporphyrin levels and were "porphyric" for 4 days or longer. These seven were therefore the most satisfactory potential experimental animals.

One of the obvious disadvantages of the present Sedormid-treated rat as an experimental animal is that the disease appears to be devastating to the animal, and frank porphyria usually lasts only about a week before death occurs. Therefore long-term experiments with the animal continuously porphyric are as yet not possible. The disease seems to kill partly by inanition, since the animals usually neither eat nor drink after frank porphyria has begun. Therefore experiments are planned in which supportive parenteral fluids and antibiotics will be used in an attempt to lengthen the course of the disorder. This situation is reminiscent of acute porphyria in humans, when patients are gravely ill during exacerbations of the disease.

C. Effect of Parenteral Sedormid Injections

The failure of parenteral Sedormid injections to cause any change in porphyrin excretion suggests that the oral route is necessary to produce the disorder. If this preliminary hypothesis proves to be true, it may mean that Sedormid must reach the liver direct via the portal system to be effective. Another possibility which should be investigated is that Sedormid exerts its effect by acting on the intestinal bacterial flora. It should be emphasized that intraperitoneal Sedormid was given to only one animal, rat 21.

D. Tissue Studies

Red fluorescence in animal tissues is a rare phenomenon, and, when seen "is presumptive evidence for the presence of porphyrins" (Turner)[26]. Thus the red fluorescence seen in the carcasses of normal and porphyric rats can be tentatively ascribed to porphyrin.

The red fluorescence seen in the central nervous system of both normal and porphyric rats proved on inspection of the literature to be a phenomenon common to all mammals. Kluver[56] described red fluorescence in the brain of 33 species of mammals (including rat) and birds, and tentatively identified the source of the fluorescence as coproporphyrin. Recently Blanchard[57] showed this porphyrin to be coproporphyrin III.

Fluorescence seen in the Harderian gland of both normal and porphyric rats has been observed in normal rats by other investigators. Isolation and identification of large amounts of protoporphyrin from the tears of rat 32 corresponds to Towbin's[58] observation that the absorption spectrum of the tears of normal rats is that of protoporphyrin.

Purple nasal and eye excretions seen in all the porphyric rats have been termed "chromodacryorrhea" by other investigators. This phenomenon has been observed in vitamin B-deficient animals, but can also be produced by water deprivation. According to Lemberg and Legge,[7] the gland excretion, which normally enters the nasolacrimal duct and is swallowed, becomes glassy and remains around the eyes and nose in water deprivation states. Because the animals cease drinking when frank porphyria occurs, their purple nasal excretions can probably be explained on this basis.

Fluorescence in the feces of normal rats perhaps can be explained by the red-fluorescent material in Purina chow. This substance has not been identified, but its solubility characteristics indicate that it is not a porphyrin. Contamination of urine with food does not give spurious porphyrin values, because this substance is not extracted with the methods employed in porphyrin analysis.

The water-soluble fluorescence in the feces of rat 33, not seen in normal animals, suggests an abnormality of fecal porphyrins in porphyric animals.

The consistent finding of a prominent red-fluorescent band seen on cut section of the kidneys of porphyric rats is thought to be a real departure from the normal. The role of the kidney in production of porphyria is not known. So far only bone marrow and liver have been implicated as principle sites of porphyrin production in porphyria.[39]

SUMMARY

The urinary porphyrin and porphobilinogen excretion of 200 gram female rats has been described.

Oral administration of Sedormid suspended in propylene glycol has caused marked increases in urinary uroporphyrin, coproporphyrin III, and porphobilinogen. These findings, coupled with concurrent clinical changes of paralysis, lethargy, and gastrointestinal dysfunction constitute a syndrome closely resembling adult human porphyria. The disorder thus produced resembles that recently described in rabbits.

Tissue studies on porphyrins of normal and Sedormid-fed rats have been described.

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