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URINARY MANIFESTATIONS OF PORPHYRIA

A DISSERTATION

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DOCTOR OF PHILOSOPHY

BY

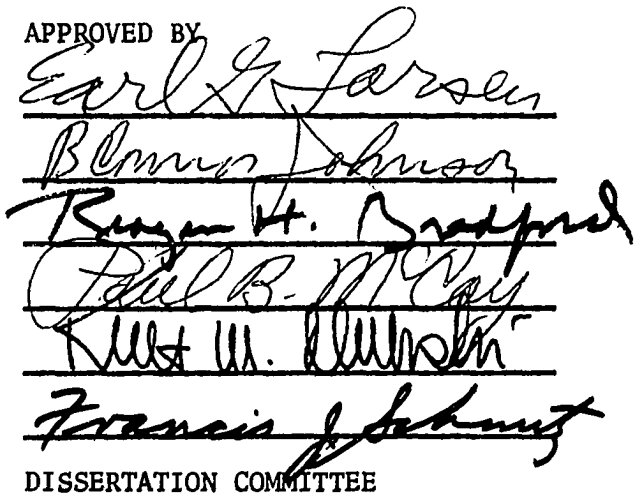
DELBERT JOE LACEFIELD

Oklahoma City, Oklahoma

1969

URINARY MANIFESTATIONS OF PORPHYRIA

APPROVED BY



DISSERTATION COMMITTEE

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URINARY MANIFESTATIONS OF PORPHYRIA

CHAPTER I

INTRODUCTION

The porphyrias are hereditary, metabolic disorders which often mimic other conditions making diagnosis difficult. The first published report of a case was by Schultz (58) in 1874 in which a wine-red urine was excreted. In the same year, Baumstark (3) isolated a pigment from this urine which was similar to heamatoporphyrin described by Hoppe-Seyler (33) in 1871. This is now known to be a case of congenital porphyria. Congenital porphyria is characterized by marked photosensitivity due to free porphyrins circulating in the blood, a wine-red urine and brown-pink teeth and bones. Large amounts of uroporphyrin I (56) are excreted in the urine and a lesser amount of coproporphyrin I (1). Uncomplicated cases do not excrete intermediates of porphyrin biosynthesis and only traces of penta and heptacarboxylic porphyrins. Coproporphyrin I predominates in the feces with small amounts of uro and protoporphyrin. The condition is extremely rare with less than 100 cases reported in the literature but is the easiest of the porphyrias to diagnose.

The most frequent of the porphyrias is acute intermittent porphyria and studies of this disease have made great contributions to the understanding of porphyrin biosynthesis. In acute intermittent porphyria there is an absence of photosensitivity but gastrointestinal and

neurological symptoms are present. The first recorded case was by Stokvis in 1889 (64). Waldenstrom (72) and Watson (77) have contributed to an understanding of the disease. Gunther (29) noted a hereditary manifestation of the disease but it was Waldenstrom who definately established the hereditary nature of the disease from his study of many affected Scandinavian families. Abnormally large amounts of delta-aminolevulinic acid and porphobilinogen are excreted in the urine and Haeger (30) has reported that these findings may persist even while the disease is in remission. The same author also reported similar findings in the latent form of the disease. Because the clinical symptoms are so non-specific a definite diagnosis is based upon the presence of abnormal concentrations of porphobilinogen in the urine.

A number of cutaneous porphyrias have been described, other than congenital porphyria, and in addition to skin lesions, all show impaired liver function. For this reason all the forms are referred to as cutaneous hepatic porphyria. The cutaneous hepatic type that is recognized as hereditary has been given various names by different authors. Thus, Waldenstrom (73) called it porphyria cutanea tarda hereditaria whereas, Watson (75) has called it mixed porphyria and Dean and Barnes (15) referred to it as porphyria variegata. It is also recognized that cutaneous hepatic porphyria may be acquired as reported by Barnes (2) among the Bantus and by Cetingil and Ozen (7) among a segment of the Turkish population. The first case of cutaneous hepatic porphyria was reported by Harris (32) in 1898. Just as the clinical symptoms are varied and contain components similar to congenital porphyria and acute intermittent porphyria so are the chemical findings. During attacks

coproporphyrin, uroporphyrin, porphobilinogen and delta-aminolevulinic acid are excreted in the urine. It is during urinary excretion of these compounds that photosensitivity appears. In remission coproporphyrin and protoporphyrin are excreted in the feces.

Large amounts of porphyrins, in excess of what is needed for normal production of the heme-containing enzymes, are found in a number of examples in the animal kingdom. Kennedy (36) extracted uroporphyrin from the shells of molluscs in large amounts. A copper complex of uroporphyrin is found in the red areas of the flight feathers of the turaco birds (48). Derrien and Turchini (16) noted an intense red fluorescence in the gland of Harder in rats. More recently, Davidheiser and Figge (14) have shown that this gland possesses the necessary enzymes to produce protoporphyrin from delta-aminolevulinic acid or porphobilinogen. In 1937, Turner (70) reported the isolation of uroporphyrin I from the urine of the fox-squirrel, Sciurus niger, and attributed the dark color of the urine to this compound. He also reported that this was unlike some other rodents who show increased pyrrole production only during fetal life. In 1955 Rimington (52) reported that the bones and teeth of the fox-squirrel fluoresce strongly in contrast to other squirrels that show only slight fluorescence. The first living case of porphyria in cattle was reported in 1936 by Fourie (20); Rimington (51); and Fourie and Rimington (21) although as early as 1884 Brouwier (5) reported pigmented bones in bovine carcass. A study of bovine porphyria shows a striking similarity to human congenital porphyria both clinically and biochemically. Cattle show pigmented teeth and bones, photosensitivity, they excrete large amounts of uroporphyrin I and copro-

porphyrin I and no porphobilinogen. In contrast to the human disease in which uroporphyrin is increased in the red cells, protoporphyrin levels are high and uroporphyrin only slightly increased in bovine red cells. A notable exception in cattle was reported in 1958 by Ellis et al. (17). In the herd they studied the urine contained porphobilinogen, uroporphyrin III and coproporphyrin III. Porphyria in pigs has also been reported by Clare and Stephens (10) and by Jorgensen and With (35).

In addition to the porphyrias, in which it is recognized that there is a primary abnormality in porphyrin metabolism, there are a large number of diseases in which porphyrin excretion is increased over normal levels. The primary disease may cause a shift from normal routes of excretion or may interfere with the normal pathway of heme biosynthesis. Table 1 summarizes the findings in some of these conditions.

The relationship of certain drugs to the porphyrias has been known for many years. The case of acute intermittent porphyria reported by Stokvis (64) was related to the taking of Sulfonal by the patient. He had earlier used this drug to produce increased excretion of coproporphyrin in the urine of rabbits (65). Barbiturates, given over long periods, may activate the latent form of acute intermittent porphyria (84) and may cause paralysis if given during an attack. Hexachlorobenzene has produced a type of porphyria (6) and is among the compounds used in recent years to produce experimental porphyria in animals (49). Schmid and Schwartz (57) used allylisopropylacetylcarbamide (Sedormid) to produce a form of porphyria in rabbits. Clinical symptoms of acute intermittent porphyria were produced and large increases in porphobilinogen, uroporphrinogen, coproporphyrinogen and

TABLE 1

PORPHYRIN ABNORMALITIES SECONDARY TO OTHER DISEASES

	Primary Disease	Porphyrin Abnormality	References
1.	Iron deficiency anemia	Increased erythrocyte and bone marrow protoporphyrin	(76)
2.	Pernicious anemia	Vitamin B_{12} causes increase in erythrocyte coproporphyrin	(74)
3.	Lead intoxication	Increase in urine delta-mainolevulinic acid and copro-porphyrin	(31)
4.	Arsenic	Increased coproporphyrin in urine	(60)
5.	p-aminobenzene compounds	Increased urinary porphyrins	(53)
6.	Sulfonamides	Increased urinary porphyrins	(54)
7.	Hexachlorobenzene	Increased urinary porphobilinogen, uroporphyrin and coproporphyrin	(6)
8.	Hepatic cirrhosis	Increased urinary coproporphyrin	(82)
9.	Infectious hepa-titis	Increased urinary coproporphyrin	(78)
10.	Alcoholism	Increased urinary coproporphyrin	(22)
11.	Cancer	Variable - depending on type	(68)
12.	Myocardial infarc-tion	Increased urinary coproporphyrin	(39)
13.	Poliomyelitis	Increased urinary coproporphyrin	(81)
14.	Rheumatic fever.	Increased urinary coproporphyrin	(46)

protoporphyrinogen were found in the liver. Porphobilinogen and uroporphyrin were increased in the urine. Goldberg and Rimington (25) used allylisopropylacetamide in rabbits and obtained essentially the same results. Coproporphyrin was also excreted by their animals. A number of barbituric acid derivatives have been used to produce experimental porphyria and Goldberg (24) found that those containing one or more allyl groups produced the greatest production of porphyrins and precursors. Diallylbarbituric acid caused the excretion of uroporphyrin, coproporphyrin and porphobilinogen. The same pattern that is produced by Sedormid and allylisopropylacetamide was produced by Solomon and Figge (63) using 2,4,6, trimethyl-1,4-dihydro-3,5, dicarbethoxypyridine. It is interesting that although there is little in common between the compounds used to produce experimental porphyria, they lead to conditions in many ways similar to those of the human porphyrias.

The foundation for the study of the porphyrias rests upon the work of Hans Fisher and his associates who synthesized many of the known naturally occurring porphyrins and other derivatives of porphin which may be considered the parent compound of all porphyrins (19). It was also Fisher who suggested that all porphyrins be referred to the four etioporphyrins, which he had synthesized, for designation of isomeric type. More recently MacDonald (41) and his group have contributed to the synthesis of the porphyrins, especially noteworthy is their synthesis of the II and IV isomers of the naturally occurring porphyrins and the first synthesis by chemical means of porphobilinogen (41, 34, 67, 45). In one or more of the porphyrias, coproporphyrin

I and III, uroporphyrin I and III and protoporphyrin IX may be found. The iron complex of protoporphyrin IX serves as the prosthetic group of all the heme containing enzymes found in nature. The reduced forms of the porphyrins may be produced by treatment with sodium borohydride (44). The porphyrinogens are easily oxidized by light, acid and iodine back to the parent compound. The porphyrinogens have been reported in the urine of patients in various disease states by Watson et al. (79), and are an important step in porphyrin biosynthesis.

The elucidation of the biosynthetic pathway to protoporphyrin is one of the outstanding accomplishments of modern biochemistry. Waldenstrom (71) recognized porphobilinogen as a distinct entity but it was not until 1952 that Westall (83) isolated the compound. Cookson and Rimington (11,12) determined that its structure was that of a B,B-dialkyl-2-aminomethylpyrrole. Using bird erythrocytes, Shemin, London and Rittenberg (62) showed that labeled glycine and succinate were incorporated into newly synthesized heme. They were able to degrade heme and account for the activity of each carbon atom. This group also synthesized delta-aminolevulinic acid and showed that it was the immediate precursor of porphobilinogen. Bogarad (4) has isolated the enzymes which produce uroporphyrinogen from porphobilinogen. Mauzerall and Granick (27) isolated an enzyme preparation from rabbit reticulocytes that converts uroporphyrinogen to coproporphyrinogen. Granick and Mauzerall also used a preparation from chicken erythrocytes that would convert coproporphyrinogen III to protoporphyrin. The preparation would not utilize coproporphyrinogen I, thus explaining the fact that protoporphyrin which would be derived from the I isomer has never been found

in nature. The general pathway of porphobilinogen biosynthesis is shown in Figure I and that of the tetrapyrroles leading to protoporphyrin is shown in Figure II.

It is the purpose of this study to elucidate the presence of intermediates in protoporphyrin biosynthesis in the urine of the fox squirrel, Sciurus niger, and in the urine of humans suffering abnormal porphyrin excretion. During the progress of this investigation a number of compounds were detected in the squirrel urine that had not previously been reported in physiological porphyrias in animals or human porphyrias. The results of this study, which are to be presented in the following pages, include the separation and isolation of various porphyrin isomers and precursors; the reactions of heretofore undescribed compounds which lead to porphyrin precursors and finally the findings in an atypical form of human porphyrinuria. A portion of the results of this study have been reported (40).

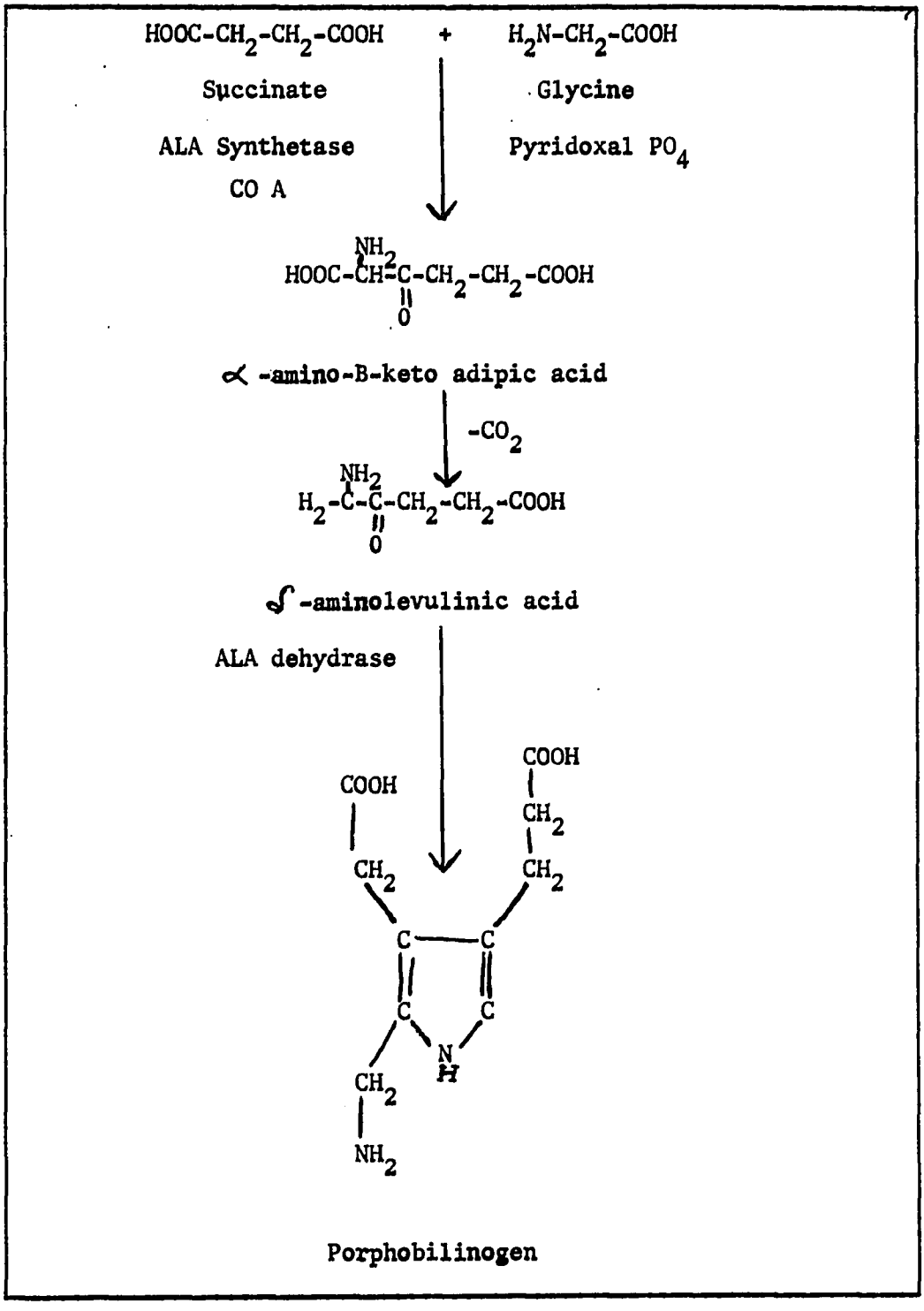


Fig. I. General Pathway of Porphobilinogen Biosynthesis

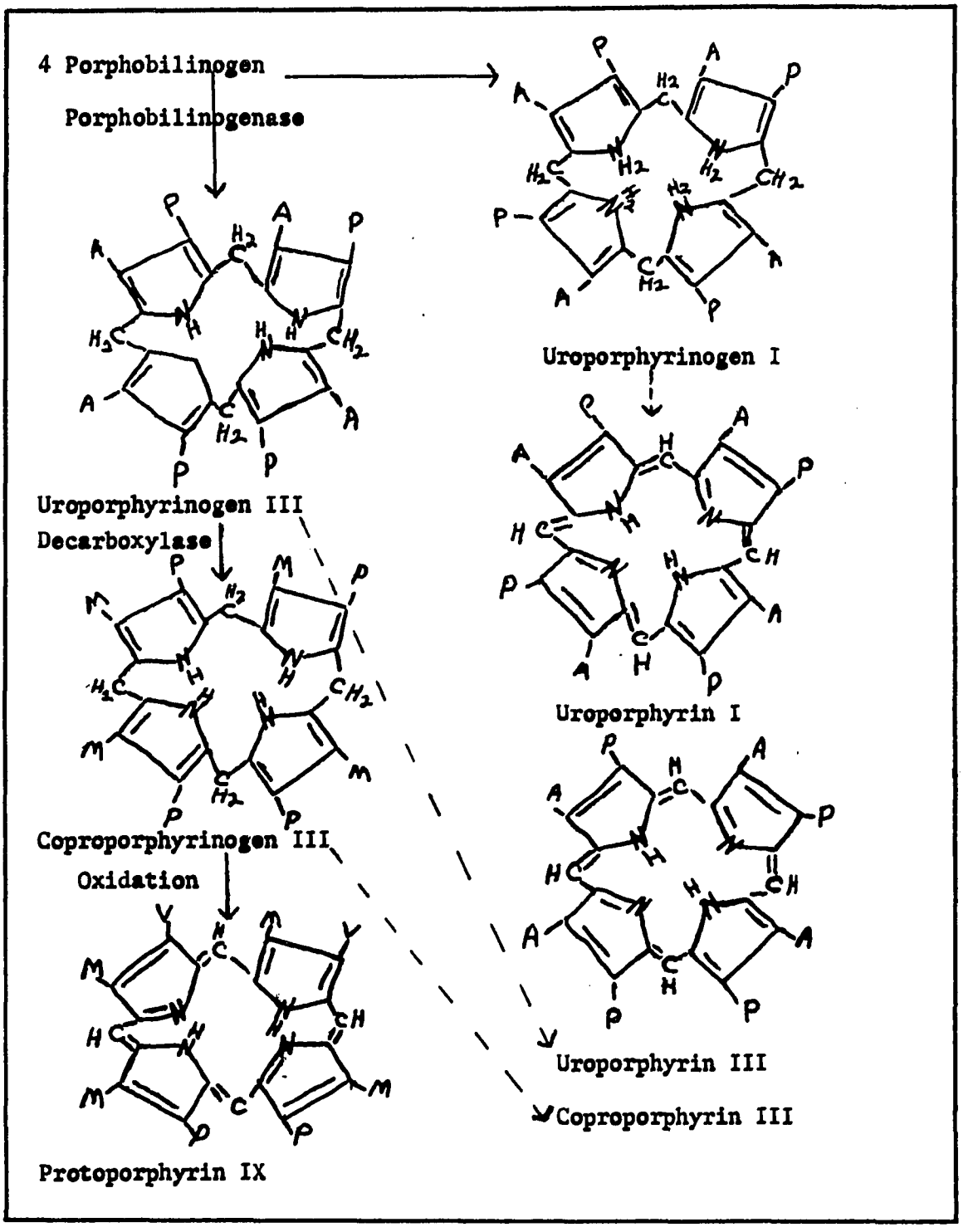


Fig. 2. General Pathway of Protoporphyrin Biosynthesis.

A = Acetic Acid, P = Propionic Acid, M = Methyl, V = Vinyl

CHAPTER II

MATERIALS AND METHODS

Materials

Fox Squirrels (Sciurus niger)

Fox squirrels were obtained from the Lincoln Park Zoo in Oklahoma City, Oklahoma, and various individuals throughout the area. The animals were maintained in metabolism cages during periods when samples of urine were collected. The cages were coated with a clear resin to minimize any metal contamination of the samples which might arise from the cages. Fine screen wire was placed over the wire mesh floor of the cages to filter small particles of food. The animals were fed a diet of nuts, grain, and rodent chow. Early in the study, animals developed a fatty infiltration of the liver and died when fed only various nuts. Animals appeared to remain normal on the mixed diet.

Squirrel Urine

Samples of squirrel urine were collected in bottles coated on the outside with black paint and containing sodium carbonate and toluene. The urine was transferred to amber bottles each day and stored in a refrigerator at 2°C until a sufficient quantity had been obtained and then it was processed.

Fresh urine was obtained by placing test tubes under the spout

of the metabolism cages and removing the water bottles from the cages in order to avoid dilution of the urine. The voiding of urine was then stimulated by exciting the animals, and fresh urine, so obtained, was immediately processed.

Squirrel Blood

Samples of squirrel blood were obtained by the following procedure. The animal, in its cage, was placed inside a large can. Ether was poured onto cotton batts and the top of the can covered with a sheet of clear plastic. As soon as the animal was anesthetized it was removed from the cage and veterinary nembutal was administered. Blood was then obtained by cardiac puncture using a size 20 needle. The syringe was coated with heparin to prevent clotting of the blood. The blood was immediately processed.

Porphyrin Methyl Esters

Protoporphyrin dimethyl ester, coproporphyrin tetramethyl ester and uroporphyrin octamethyl ester were obtained from the collection of Dr. Earl G. Larsen which had been isolated from natural sources.

S-Aminolevulinic Acid

This material was obtained as a gift from Dr. Black of the Parke Davis Pharmaceutical Company, Detroit, Michigan.

Porphobilinogen

This material was obtained as a gift from Dr. S. F. McDonald, Canadian Research Council, Ottawa, Canada.

Silicic Acid

Silicic acid prepared for chromatographic analysis was purchased from Mallinckrodt Chemical Works, St. Louis, Missouri.

Dowex 2-X8 Resin

Dowex 2-X8 resin of 200 to 400 mesh in the chloride form was purchased from the J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Dowex 50-X8 Resin

Dowex 50-X8 resin of 200 to 400 mesh in the hydrogen ion form was purchased from the J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Methods

Determination of Porphobilinogen

Porphobilinogen was determined by the method of Mauzerall and Granick (43). Porphobilinogen is retained on Dowex 2 resin in the acetate form. The resin, as obtained, was in the chloride form and was converted to the acetate form by suspending it in 3N sodium acetate and stirring on a magnetic stirrer. The material was then allowed to settle and the supernatant decanted off. This procedure was repeated until the supernatant was free of chloride ions as shown by a failure to give a precipitate with silver nitrate. The resin was then washed with several volumes of distilled water to remove any excess sodium acetate. This resin was stored in amber bottles until used and was stable for several

months.

Urine was adjusted to a pH between 5 and 7 with 3N hydrochloric acid and 2 mls were pipetted onto a column of Dowex 2-X8 resin (1.0 x 2.5 cm) and allowed to drain. Two 2 ml portions of water were added to the column and the urine eluate and washings were collected for delta-aminolevulinic acid determination. Porphobilinogen was eluted from the column into a 10 ml volumetric flask by adding 2 mls of 1.0 N acetic acid followed by 5 mls of 0.2 N acetic acid. The acid eluate was diluted to 10 mls with distilled water. To a 2 ml aliquot of the porphobilinogen solution was added 2 ml of a modified Ehrlich's reagent. The mixture was placed in a cuvette and the optical density at 555 mu was determined 15 minutes after mixing. The modified Ehrlich's reagent was prepared by dissolving 0.5 gm. of p-dimethylaminobenzaldehyde in about 15 ml of glacial acetic acid, 4.0 ml of 70 per cent perchloric acid was added, and the solution diluted to 25.0 ml with acetic acid. This reagent was prepared fresh each day.

Delta-aminolevulinic Acid Concentration

To a column of Dowex 2-X8 resin (1.0 x 2.5 cm.) 2.0 ml of urine at pH 5 to 7 was added and allowed to drain. The resin was washed with two 2.0 ml portions of water and the combined eluate was quantitatively transferred to a column of Dowex 50-X8 resin (1.0 x 2.5 cm.). After draining, the column was washed with 16 mls of water to remove urea. The elimination of urea was followed on a spot plate by adding a drop of Ehrlich's reagent to a drop of the washing. As long as urea was present in the washings, as shown by a yellow color when reacted

with Ehrlich's reagent, the washing was continued. 3.0 ml of 0.5 N sodium acetate was passed through the column and then the delta-aminolevulinic acid was eluted with 7.0 mls of 0.5 N sodium acetate. The delta-aminolevulinic acid was converted to 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole by adding 0.2 ml of 2,4-pentanedione and diluting the solution to 10.0 ml with a pH 4.6 acetate buffer. This solution was placed in boiling water and heated for ten minutes. After cooling to room temperature, a 2.0 ml aliquot was reacted with 2.0 mls of the modified Ehrlich's reagent. After 15 minutes the optical density at 553 mu was read against a water blank treated in the same manner as the Dowex 50 eluate.

The Dowex 50 resin was prepared from commercial samples of resin by converting it to the sodium form by allowing it to stand overnight in 2 N sodium hydroxide. The resin was then washed until neutral, followed by one volume of 4 N, six volumes of 2 N and six volumes of 1 N hydrochloric acid and six volumes of water. The resin was stored under water in amber bottles.

Isolation of Porphyrins from Urine

Phosphate absorption. Porphyrins were isolated from squirrel urine by a modification of the method of Corwin and Orten (13). Pooled urine samples that had been stored in amber bottles in a refrigerator at 2°C were thoroughly mixed and the pH adjusted to 4.5 with a 4-1 mixture of glacial acetic acid and saturated sodium acetate solution. A saturated solution of lead acetate was slowly added to the urine until no further precipitate formed in the supernatant of a portion of the mix-

ture that had been centrifuged. The solution was stirred on a magnetic stirrer for several hours to insure complete absorption of the porphyrins on the phosphate precipitate. The solution was centrifuged and the supernatant was discarded. The precipitant was washed twice with water and once with methyl alcohol.

The porphyrins were extracted from the precipitate and simultaneously converted to the methyl esters by refluxing the precipitate for 15 minutes with an anhydrous methanol-hydrochloric acid mixture. The mixture was again centrifuged and the supernatant, containing the prophyrin esters, was poured into chloroform. The extraction and esterification was continued until the supernatant obtained after centrifuging failed to fluoresce red when exposed to 3660A° ultraviolet light.

The solution of porphyrins in chloroform was adjusted to a pH of 4.5-5.0 with a saturated sodium acetate solution. The chloroform layer was removed and washed repeatedly with water until the washings were neutral. Solid anhydrous sodium sulfate was added to the chloroform to remove the last traces of water. The sodium sulfate was filtered from the chloroform and washed with chloroform until all fluorescent material was removed from the sodium sulfate. The filtrate was evaporated to dryness, on a turning evaporator, under reduced pressure and at elevated temperatures. The dried material containing the porphyrin esters was stored in the dark until fractionated.

Ion Exchange Resin. Pooled urine was adjusted to a pH of 4.5-5.0 with an acetate buffer. Dowex 1 resin in the chloride form was slowly added to the urine solution with constant stirring. The mixture

was stirred for several hours and set in the refrigerator overnight. The resin was then centrifuged down and the supernatant saved for furthur analysis. The resin was washed several times with water and absolute methanol. The porphyrins were esterified and eluted from the resin with an anhydrous methanol-hydrochloric acid mixture. The solution containing the porphyrin esters was then treated in the same manner as described in the section on phosphate absorption.

Column Chromatography of Porphyrin Ester Mixtures

Crude mixtures of porphyrin methyl esters and other material were fractionated using columns of hydrated silicic acid. Silicic acid as purchased contained varying quantities of water which made it impossible to control the degree of hydration of the silicic acid. Therefore, the silicic acid was dried over phosphorus pentoxide in a vacuum oven at 60° centigrade until no moisture was evident in the phosphorous pentoxide. This material was stored over the same desicant in a vacuum desicator until used.

Columns of silica gel were prepared by hydrating previously dried silicic acid with thirty percent water in a beaker. The powder was stirred vigorously and pressed against the side of the beaker to insure thorough and uniform hydration. During the hydration, much heat was evolved. After hydration, hexane was added to the gel and the slurry was poured into a glass column containing a glass wool plug at the bottom. The slurry was stirred in the column to rid it of any trapped air. The column was then packed by tapping the lower end with a wooden block as positive air pressure was applied to the top of the

column. Even packing was fascilitated by dividing the silica gel into several portions and packing each thoroughly before adding the next portion. A smaller portion of the gel was used to absorb the porphyrin esters from a hexane solution containing just sufficient chloroform to effect solution. The gel containing the absorbed porphyrin esters was then poured on top of the packed column and the column was filled with hexane. This last amount of gel was packed in the same manner as the remainder of the column. Development of the chromatogram was begun and continued with various proportions of chloroform in normal-hexane.

Paper Chromatography of Free Porphyrins

Ascending paper chromatography was run on Whatman No. 1 filter paper using 2, 6-lutidine-cyclohexanone-water as solvent. The chromatography was allowed to run for 9 to 10 hours in the refrigerator at 1 to 2° centigrade. The porphyrins were located on the wet paper by their fluorescense under a 3660A° ultraviolet lamp. Authentic porphyrin samples were run on the same paper each time for comparison with the unknowns.

Paper Chromatography of Porphyrin Methyl Esters

Two dimentional paper chromatography was run on 19 cm squares of Whatman No. 1 filter paper which was rolled into a cylinder and stapled. First, a solvent system of kerosene and chloroform was run until the solvent front advanced approximately 15 cm. Chloroform was placed in the bottom of the chamber to saturate the atmosphere. The paper was dried at 105-110°C for 4 minutes and then placed in a solvent of kerosene and normal porpanol in a kerosene atmosphere. The solvent

was allowed to migrate the same distance as the first solvent. The paper was again dried briefly at 105-110°C. The paper was turned 90 degrees and placed in a solvent system of kerosene and p-dioxane. This solvent was run to the top of the paper. During this development the atmosphere was saturated with dioxane. The entire development of the chromatogram was run at room temperature. The porphyrin esters were located on the paper by their fluorescense under a 3660A° ultraviolet lamp.

Paper Chromatography of δ-aminolevulinic acid

Delta-aminolevulinic acid was chromatographed on Whatman No. 1 paper. Two dimensional chromatograms were run at room temperature. The first solvent was n-butanol-glacial acetic acid-water (63:27:10) and was run for 3 to 4 hours. The paper was dried at room temperature and turned 90 degrees and run for 8 hours in a solvent system of phenol and water (100 grams of phenol and 39 ml of water). The chromatogram was dried at room temperature until the phenol odor was not detectable. The spots were located by spraying the paper with a 0.5 percent solution of ninhydrin in n-butanol saturated with pyridine-glacial acetic acid and water (10:1:95). δ-aminolevulinic acid appeared as a yellow spot after heating for a few minutes at 60°C.

Paper Chromatography of 2-Methyl-3-Acetyl-4-(3-Propionic acid) Pyrrole

This pyrrole was chromatogramed on Whatman No. 1 paper in a solvent system of n-butanol and acetic acid. The solvent was prepared by mixing in a separatory funnel 4 volumes of n-butanol, 1 volume of

acetic acid, and 5 volumes of water. The solution was mixed and after complete separation the upper phase was used as solvent. The solvent front was allowed to migrate, at room temperature, to near the top of the paper. The spot was then located by running a modified Ehrlich's reagent (see section on porphobilinogen determination) onto the paper from a pipette. A pink spot appeared which rapidly faded.

Preparation of 2-Methyl-3-Acetyl-4-(3-Propionic acid) Pyrrole

An aqueous solution of 50 mg of δ-aminolevulinic acid and 0.2 ml of 2,4-pentanedione and 20 ml of pH 4.6 acetate buffer was heated in a boiling water bath for 10 minutes. The solution was cooled to room temperature and the pH adjusted to 7.0. The excess 2,4-pentanedione was removed by shaking the solution with chloroform several times. The chloroform was removed from the aqueous solution by bubbling air through it until the chloroform odor was not detected. The pH of the solution was then adjusted to 1 with dilute hydrochloric acid and the solution set in the refrigerator. After several hours, crystals had formed and the solution was left overnight. The crystals were filtered, washed and dried over phosphorus pentoxide in vacuo. The crystals were white and gave a strong Ehrlich's reaction. The melting point was 191-193° against a reported 194° (43).

Determination of Reticulocyte Percentage

Blood was obtained by cutting the end of the tail of the squirrel and mixing one drop of fresh blood with one drop Brilliant Cresyl Blue stain. After standing one minute, a thin smear was made and allowed to dry. The smear was then counterstained with Wrights'

stain. The slide was scanned under oil and 1000 red blood cells counted and the number of reticulocytes recorded.

Determination of Porphyrinogens

Porphyrinogens were determined by a modification of the method of Schwartz *et al.* (61) on freshly voided urine. To a 10 ml. sample, 5 mls of a 4:1 glacial acetic acid-saturated sodium acetate solution were added. 125 mls of ethyl acetate and 30 mls of water were next added to the acidified urine. The mixture was vigorously shaken in a separatory funnel and the aqueous layer discarded. The organic phase was washed twice with 25 mls. of 1 percent sodium acetate. The aqueous layer was collected and viewed under an ultraviolet light. The organic phase was next washed with a 0.005 percent iodine solution to convert the porphyrinogens to porphyrins. The iodine wash was discarded and the porphyrins extracted from the ethyl acetate by repeated extractions with 5 ml. portions of 1.5 N hydrochloric acid. The hydrochloric acid extractions were continued until they became colorless and showed no red fluorescence. Preformed prophyrins were extracted from a second sample by the same method, except the iodine wash was omitted.

CHAPTER III

RESULTS

Isolation of Porphyrins from Urine

To determine if age or sex was a factor in the type of porphyrins excreted in the urine of the squirrel, separate urine samples were collected from mature and juvenile male and female squirrels. Collection was carried out as described in the previous section on methods until 350 to 500 mls. had been accumulated. The pH of the urine was adjusted to between 4.0 and 4.5 with a 4:1 mixture of acetic acid and saturated sodium acetate solution. It was found that 6 mls. of this solution per 100 mls. of urine were usually sufficient. Upon addition of saturated lead acetate a heavy white precipitate formed. Approximately 15 mls. of lead acetate per 100 mls of urine caused complete precipitation. The solution was stirred for several hours and refrigerated overnight. Under these conditions, the original dark brown urine became much lighter and the porphyrins were absorbed on the precipitated material. The solution was again mixed and centrifuged for several minutes at 2000 r.p.m. The supernatant was set in the refrigerator for further study. After washing twice with distilled water, the precipitate was washed with absolute methanol. After centrifuging, the methanol supernatant was pink but did not fluoresce under ultraviolet light. A second methanol wash was yellow and the third methanol wash

was bright blue. After four washings, the supernatant was clear. The colored pigments extracted by methanol washings were discarded and no attempt was made to identify them.

Absolute methanol containing dissolved hydrochloric acid was added to the washed precipitate and the mixture placed in a water bath heated slightly above the boiling point of the mixture. Boiling was continued for 30 minutes during which time the solution was occasionally agitated. The mixture was then centrifuged and a maroon supernatant was obtained which fluoresced a brilliant red upon exposure to ultraviolet light. The supernatant was poured into chloroform and further esertification and extraction performed on the precipitate until the supernatant solution was no longer red and failed to fluoresce.

The combined methanol-hydrochloric acid extract of the lead acetate precipitate, in chloroform, was neutralized with saturated sodium acetate to a pH of 4.0 to 5.0. At this pH the porphyrin esters dissolved in the cloroform. Additional chloroform was added to the solution and all the red fluoresing material was found to have separated into the chloroform. The chloroform layer was separated and washed repeatedly with distilled water until the washings were no longer acid as indicated by pHydrion paper. Anhydrous sodium sulfate was added to the chloroform to remove water from the solution. After standing overnight, the sodium sulfate was filtered and washed with chloroform until all fluorescent material was removed. The washings and filtrate were combined and evaporated to dryness under reduced pressure on a turning evaporator. The dried residue was deep brown-red color.

The crude mixture of porphyrin methyl esters was fractionated using columns of hydrated silicic acid which were prepared as described in the section on methods. Each column contained silica gel made by mixing 120 grams of silicic acid with 36 mls. of water. The final dimensions of the packed columns were 43 by 180 millimeters. After the porphyrin mixture was added to the columns, normal-hexane was added and 200 mls. allowed to pass through the column to insure complete adsorption of any porphyrin that was dissolved in the column of liquid above the top of the silica gel. Development of the chromatogram was started with a solution of one part chloroform in three parts hexane. With 450 to 500 mls. of solvent above the packed column, a flow rate of approximately 40 ml. per hour was obtained. Three hundred mls. of the initial solvent mixture passed through the columns and it was noted that a yellow band was moving down the column followed by a red band and one of a blue-violet color. None of these zones showed any fluorescence. The concentration of chloroform was increased to thirty per cent and the column was fitted with a reservoir of sufficient size to hold enough solvent to permit a continuous flow overnight. During this interval, approximately 500 mls. of solvent passed through the columns and a red fluorescent band was one centimeter below the origin. The solvent was changed to forty per cent chloroform and development continued until the first fluorescent zone was about two centimeters below the origin. As each fraction migrated down the column, the ratio of chloroform to hexane was gradually increased. After most of the fluorescent material was removed from the column, 100 per cent chloroform was added which removed most of the remaining red fluorescent compounds.

Those not removed by chloroform could be removed by a 2 to 5 per cent solution of acetic acid in chloroform.

It was possible to follow the migration on the fractions on the column visually only if the zone was concentrated or very large. However, even when a fraction could not be detected visually, it was possible to visualize the smaller fractions by shining the ultraviolet light on the column. This not only made it possible to detect minor fractions but was the only method found suitable for determining if a fraction had been completely eluted from the column and collected.

As each fraction was eluted, it was collected and evaporated to dryness under reduced pressure on a rotary evaporator. The solid material was re-dissolved in a minimal amount of chloroform and transferred to small flasks. The solution was again evaporated to dryness and stored in a dark cabinet.

After several such columns had been developed, starting with material isolated from various animals it became apparant that a similar pattern of results was obtained regardless of age or sex. This pattern can be seen in Figure 3 and Table 2.

Tentative identification of the material in each fraction was made by determining the absorption maxima of the material dissolved in chloroform using a Bausch and Lomb Spectrometer. The absorption maxima are shown in Table 3. Three separate readings were made on each sample by two people. The following absorption maxima for the porphyrin methyl esters in chloroform were reported by Fisher and Orth (19). Corproporphyrin 622, 567, 532, 500, 405; Uroporphyrin 626, 571, 535, 502, 408; Protoporphyrin 631.3, 576, 541.6, 507.7.

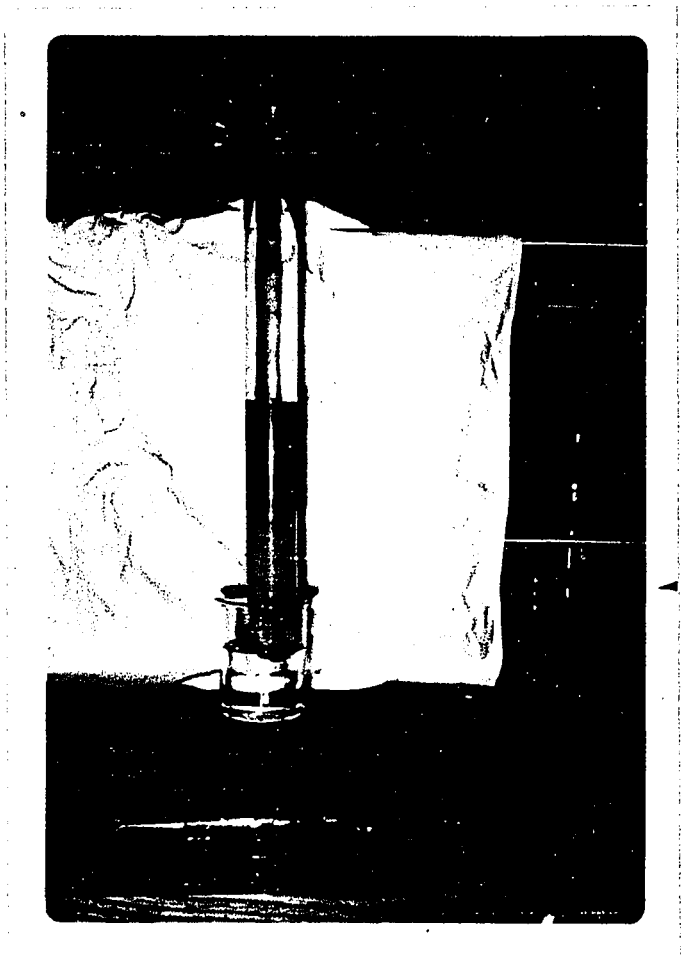


Fig. 3. Chromatography of Porphyrin Methyl Esters on a Silica Gel Column.

TABLE 2

ELUTION PATTERN OF PORPHYRIN METHYL ESTERS
FROM SILICA GEL COLUMNS

Column I

Fraction	Number and Color of Bands	Fluorescence
1	1 yellow, 1 red, 1 violet	Negative
2	2 red (one zone was heavy and the second small and narrow)	Positive
3	1 red (diffuse)	Questionable
4	2 red (small zone just ahead of large zone)	Positive
5	1 pink (diffuse)	Positive
6	1 red-brown (narrow, sharp band)	Positive
7	1 red	Positive

Column V

Fraction	Number and Color of Bands	Fluorescence
1	2 yellow, 2 red, 1 violet	Negative
2	2 red (one large and one small zone)	Positive
3	1 red (diffuse)	Questionable
4	1 red (very large)	Positive
5	1 pink (diffuse)	Positive
6	1 red-brown (narrow, sharp band)	Positive
7	1 red	Positive

TABLE 3

SPECTROMETER READINGS OF FRACTIONS FROM SQUIRREL URINE

Fraction	Maximum Absorption			
1		563	527	
2	622	567	532	498
3		566	528	
4	626	567	535	503
5		565	527	
6	625	567	533	503
7		566	528	

From these results, it appeared that both coproporphyrin and uroporphyrin were isolated from the urine. A number of the fractions which fluoresced showed a complete absence of absorption in the 620 to 630 region which is one of the characteristic absorption bands of the porphyrins. Authentic samples of coproporphyrin and protoporphyrin were examined at the same time as the unknown samples. The amount of material in each fraction was very small, in no case exceeding a few milligrams. Silica gel columns of the size employed do not separate the isomers of the individual porphyrin types. It is not possible by absorption maxima to determine if more than one type of isomer is in solution. Neither is it possible to know the purity of the sample.

Although the maximum absorption readings showed that fraction 2 appeared to be coproporphyrin and fraction 4 appeared to be uroporphyrin, reference to Table 2 shows that fraction 2 from Columns I and V

contained two bands that could not be separated at the time of removal from the column. Likewise, reference to Table 2 shows that fraction 4 from Column I also contained two bands. The corresponding fraction from Column V was so dark and large that it possibly contained more than one substance.

In order to obtain larger quantities of material with which to work, several batches of urine were processed. With volumes of urine up to 700 mls. a pattern almost identical to that already described was obtained on the silica gel columns. However, when volumes of 3000 mls. were employed, a number of additional zones were resolved on the columns. By very gradually increasing the concentration of chloroform in hexane as the column developed it was possible to obtain as many as thirteen distinct fractions. As can be seen in Table 4, the same components as obtained from earlier columns are present and additional reddish fluorescent fractions are also present.

The material represented in Table 4 was isolated from urine which had been adjusted to pH of 4.5-5.0 with an acetate buffer by slowly adding Dowex 1 resin in the chloride form. The mixture was stirred for several hours and then refrigerated overnight. The resin was centrifuged off and the supernatant saved. The resin was washed with water and methanol in the same manner as the previously described lead phosphate precipitate. The methanol washes from the resin did not show the yellow, red and blue color as those from the lead phosphate precipitates. The material on the resin was esterified and further treated as previously described for the phosphate adsorption.

TABLE 4

ELUTION PATTERN OF PORPHYRIN METHYL ESTERS FROM SILICA GEL CHROMATOGRAPHY OF 3000 MLS. URINE

Fraction	Number and Color of Bands	Fluorescence
1	1 yellow band	Negative
2	1 red and 1 violet band	Negative
3	1 red band	Negative
4	1 orange-red band	Negative
5	1 red band	Positive
6	1 red-maroon band	Positive
7	1 red-maroon band	Positive
8	1 pink-red band	Positive
9	1 orange-red band	Positive
10	1 red band	Negative
11	1 red-brown band	Negative
12	1 red-brown band	Positive
13	1 red band	Positive

Since a greater number of fractions was obtained upon chromatography of the material isolated in this fashion it became necessary to determine if this were due only to the larger volune of urine used which would give a greater concentration of minor components or if the lead acetate precipitation was removing only a portion of the porphyrins present in the urine. This was checked by treating urine in the

usual way with lead acetate and after centrifuging down the precipitated material, adding Dowex 1 resin to the supernatant. After several hours of stirring and standing overnight, the resin was washed and any adsorbed material eluted with a methanol-hydrochloric acid mixture. The methanolic solution was separated by centrifugation and failed to show any fluorescence under an ultraviolet light. It appeared that the greater number of fractions obtained from the larger volume of urine was not due to incomplete removal by lead acetate but rather due to the fact that some of the fractions were present in such low concentration that they could not be detected on small volumes of urine.

To the supernatant solution obtained after urine had been treated with Dowex 1 resin, lead acetate was added and the resulting precipitate treated in the usual manner. After esterification and elution from the precipitate, the resulting solution showed a distinct fluorescence thus indicating that the resin had not removed all of the porphyrin material from the urine. This further substantiated the belief that some of the porphyrins were present in such low concentration that they could not be detected in small volumes of urine. Throughout the remainder of the study, lead acetate was used to form an adsorbing precipitate for the removal of porphyrins from urine.

For several months urine was collected from four animals of the same age and pooled. After a volume of 1200 mls. to 3000 mls. was obtained, the porphyrins were fractionated on columns and tentatively identified by their adsorption maxima. After a number of samples had been obtained having the same spectra, they were pooled in order to further purify the relatively crude fractions.

Fractionation of Uroporphyrin Esters

A column was prepared by hydrating 120 grams of dried silicic acid with 60 mls. of distilled water and packing it with slight air pressure. Those fractions having a high content of uroporphyrin were dissolved in a minimal amount of chloroform and poured into a slurry of 20 grams silicic acid hydrated with 10 mls. of water and several hundred milliliters of hexane. The slurry was stirred until the porphyrin esters were adsorbed on the silica gel. This mixture was carefully added to the packed column and development started with 30 per cent chloroform in hexane. 400 mls. of this solvent mixture was passed through the column in approximately 10 hours. The concentration of chloroform was increased to 40 per cent and developed overnight with an additional 400 mls. having passed through the column. The next morning, a small red band had migrated the length of the column but it was non-fluorescent. Fifty per cent chloroform was added to the column and no appreciable movement of the large zone at the top of the column occurred after passage of 200 mls. The concentration of chloroform was gradually increased to 60 per cent and the column allowed to develop overnight. The large zone moved with this concentration and several very small zones were eluted well ahead of the large zone and discarded. As the large zone moved down the column, it became wider with a diffuse front, a very dark concentrated center, and a less concentrated tailing area. As the material was eluted from the column, six fractions were collected. The first fraction was the diffuse front, the second fraction was the first half of the concentrated center section, the third

fraction was the second half of the center section and fractions four, five, and six were from the diffuse tailing area. For complete elution 800 mls. of 60 per cent chloroform solution was required. The division of the fractions is shown in Figure 4.

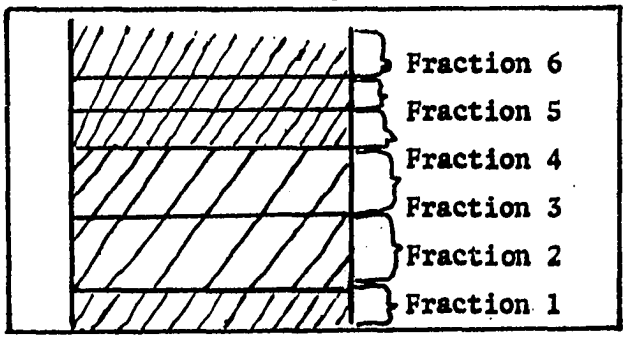


Fig. 4. Collection of fractions from the purification of crude uroporphyrin methyl esters.

Each fraction was evaporated to dryness and dissolved in chloroform and the absorption spectra determined. The results are shown in Table 5.

TABLE 5

SPECTROMETER READINGS OF FRACTIONS OBTAINED FORM THE PURIFICATION OF CRUDE UROPORPHYRIN METHYL ESTER

Fraction	Maximum Absorption		
1	---	569	536
2	626	567	535
3	626	568	536
4	626	566	533
5	626	566	534
6	626	565	535

Approximately 40 mg of material was initially placed on the column. Fractions 1, 4, 5 and 6 combined contained about one-third of the recovered material. Fractions 2 and 3 each contained about one-third of the material recovered. A trace of the starting material remained at the top of the column and was not recovered.

Further purification of the uroporphyrin fractions on silica gel was not necessary for the crystallization of the uroporphyrin isomers. Uroporphyrin I is easily crystallized from fractions high in this isomer using chloroform-methanol with any uroporphyrin III that might be present remaining in the supernatant. Fraction 2 was evaporated to dryness and redissolved in a small amount of warm chloroform and boiling methanol added slowly until the concentration of chloroform in methanol was 40 to 50 per cent. The solution was then heated on a boiling water bath until the first appearance of needle-like crystals were observed. The flask was stoppered and allowed to cool to room temperature and remain at this temperature for several hours. The crystals were filtered and washed with 25 to 30 per cent chloroform in methanol. After two or three crystallizations by the method described, the crystals were dried over phosphorus pentoxide in a vacuum desicator. The melting point of the dried crystals was 290-291° as determined on a Fisher-Johns Melting Point Apparatus. This agrees well with that obtained by Rimington and Miles (56) for uroporphyrin I.

A portion of the dried crystals was dissolved in chloroform and the continuous absorption spectrum determined utilizing a Cary Model 14 Recording Spectrophotometer. The spectrum obtained can be seen in Figure 5.

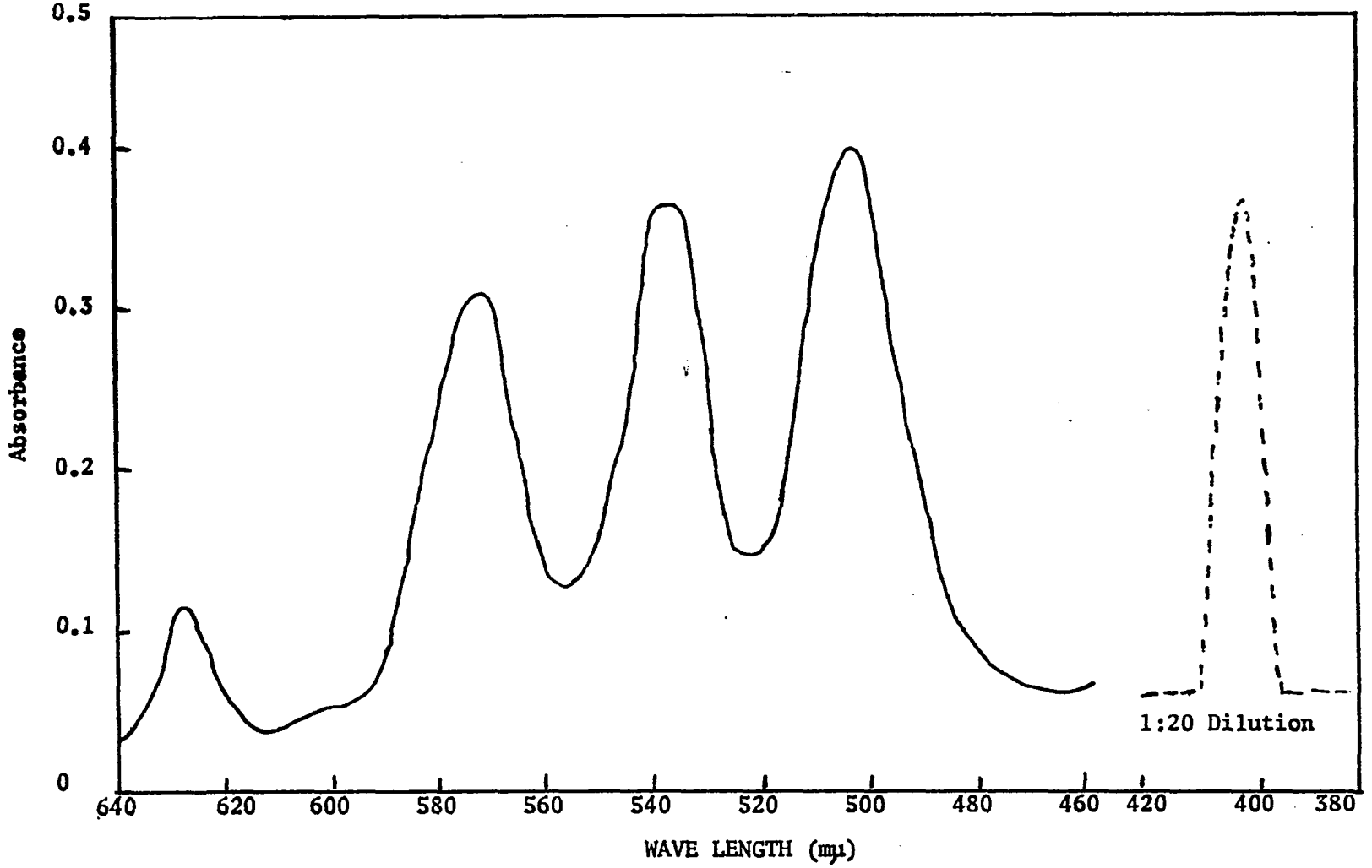


Fig. 5. Absorption spectrum of recrystallized Uroporphyrin I octamethyl ester isolated from squirrel urine.

Fraction 3 was evaporated to dryness and solubilized in warm chloroform. Boiling methanol was carefully added until a solution of 20 to 30 per cent chloroform in methanol was achieved. The solution was heated on a boiling water bath until needle-like flocculent crystals could be observed. It was necessary to heat the solution until the volume was decreased an appreciable amount. The flask was stoppered and allowed to remain at room temperature for several hours and then refrigerated overnight. The crystals were filtered and washed several times with 20 per cent chloroform in methanol. The dried crystals were again crystallized by the same prodedure. The resulting crop of crystals was dissolved in hot benzene, aided by a few milliliters of chloroform. The solution was heated on a water bath until the odor of chloroform could not be detected. Petroleum ether (30-40°) was slowly added to the hot benzene solution until the first formation of crystals was observed. The solution was then warmed and the crystals returned to solution. The flask was then stoppered and allowed to stand at room temperature for several hours and in the refrigerator overnight. The crystals which formed were filtered and washed with 30 per cent benzene in petroleum ether. After four additional crystallizations from benzene and petroleum ether, the dried crystals melted at 263-264° which is practically identical to the melting point of 264° reported by Nicholas and Rimington (47) for uroporphrin III. The absorption spectrum of the crystals was identical to that obtained on the uroporphyrin I sample (Figure 5).

Fractionation of Coproporphyrin Esters

A column was prepared from 60 grams of dried silicic acid hydrated with 12 mls. of distilled water. The silica gel was suspended in hexane and poured into a column of 18 mm. and packed with slight air pressure. Fractions which had shown a peak absorption between 622.0 and 622.5 were dissolved in chloroform and poured into a hexane slurry of 10 grams of silicic acid hydrated with 2 mls. of distilled water. The slurry was stirred until the porphyrin esters were adsorbed on the silica gel. The mixture was carefully layered on top of the packed column and development started with 30 per cent chloroform in hexane. As the concentration of chloroform was gradually increased, several small bands of red non-fluorescent material migrated away from the principle area of fluorescence. Essentially all of the material placed on the column migrated as one wide band when the chloroform concentration reached 55 to 60 per cent. The large area was divided into five fractions as it was eluted from the column in the same manner as described for the uroporphyrin esters. Each of the five fractions showed an atypical orange-pink fluorescence and no longer possessed an absorption band in the 620 to 630 region of the spectrum. From this finding, it appeared as if the coproporphyrin fractions were unstable in the dark at room temperature.

Further material was isolated from urine and that which was predominately coproporphyrin was immediately re-chromatographed on an 18 mm column as described. However, the freshly isolated material showed a typical porphyrin fluorescence and migrated with a concentration of 40 per cent chloroform in hexane. The principle band was

divided into six fractions by the method described.

Each fraction was evaporated to dryness, dissolved in chloroform, and the absorption spectra determined. The results are shown in Table 6.

TABLE 6

SPECTROMETER READINGS OF FRACTIONS OBTAINED FROM THE PURIFICATION OF CRUDE COPOROPORPHYRIN METHYL ESTER

Fraction	Maximum Absorption			
1	623	568	531	501
2	622.5	567	532	500.5
3	623	568	531.5	501
4	623	567	532	501
5	623	569	534	---
6	623	566	533	---

These data represent material which was isolated from urine and purified twice by chromatography within a period of two weeks. This coproporphyrin did not decompose as the fractions stored for long periods.

Fraction 2, Table 6, was evaporated to dryness and dissolved in a minimal amount of warm chloroform. Boiling methanol was slowly added until a solution of 60 per cent methanol was obtained and the mixture was heated on a boiling water bath until needle-like crystals appeared. The flask was stoppered and allowed to cool to room tempera-

ture. After several hours, the crystals were filtered on a sintered glass filter and washed with 15 per cent chloroform in methanol. The crystals on the filter were again dissolved in warm chloroform and crystallized by the same procedure. After three crystallizations, the material was dried over phosphorus pentoxide in a vacuum desicator. The dried crystals melted at 251-252°. This agrees well with the melting point of coproporphyrin I of 253° reported by Nicholas and Rimington (47).

A portion of the dried coproporphyrin I crystals was dissolved in chloroform and the absorption spectrum determined using a recording spectrophotometer. The spectrum is shown in Figure 6.

The supernatants and washings obtained from the coproporphyrin I fraction were added to fraction 3, Table 6, as these washings usually contain some coproporphyrin III. The combined material was evaporated to dryness and again chromatographed on a silica gel column of 10 mm. in diameter. The material migrated as one dark zone in 40 per cent chloroform in hexane with a light diffuse front and a slight tailing of red fluorescent material. Only the heavy dark zone was collected, the front and tailing area being discarded. This material was evaporated to dryness and dissolved in warm chloroform and boiling methanol added to dissolve the crystals and the mixture again heated until the volume was decreased to a point near which the first flocculation occurred. The flask was then stoppered and allowed to cool to room temperature and placed in the refrigerator overnight. The crystals were washed several times with 10 per cent chloroform in methanol. After two further crystallizations, a material was obtained which melted at

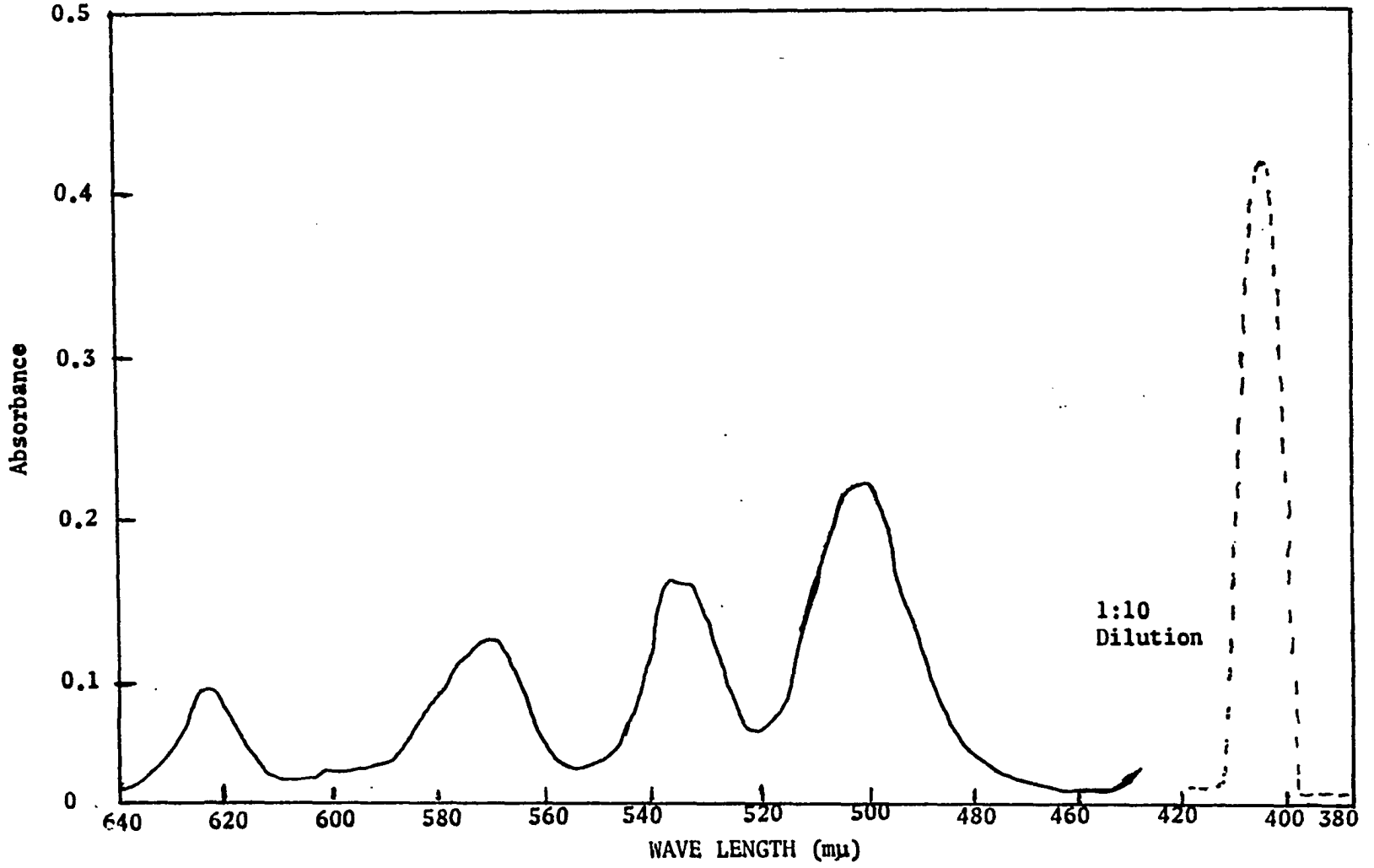


Fig. 6. Absorption spectrum of recrystallized Coproporphyrin I tetramethyl ester isolated from squirrel urine.

162-165°.

The crystalline material obtained from chloroform and methanol was dissolved in hot benzene, aided by a small amount of chloroform. The solution was then heated on a boiling water bath until chloroform could no longer be detected in the vapors. Petroleum ether (30-40°) was slowly added to the warm benzene solution until the appearance of needle-like crystals. The flask was stoppered and allowed to cool to room temperature and after several hours placed in a refrigerator. The resulting crystaline material was filtered and washing with 20 per cent benzene and petroleum ether. After three similar crystallizations the dry crystals melted at 175 degrees. Rimington and Miles (56) reported that Coproporphyrin III melted at 155° and remelted at 175° with the first melting point difficult to observe. Portions of the dryed crystals were dissolved in chloroform and the resulting absorption spectrum was identical to that of Coproporphyrin I (Figure 6).

Paper Chromatography of Free Porphyrins

In an effort to further identify the material obtained from the squirrel urine by column chromatography the technique of ascending chromatography was used. The method of Corwen and Orten (13) was utilized. This consisted of fitting a large test tube of approximately 20 ml. in diameter with a rubber stopper which had been slit crosswise and into this slit was introduced a piece of Whatman No. 1 filter paper which at the bottom contained a small glass rod to keep it suspended vertically in the test tube. A sample of free porphyrin was applied to the paper and allowed to dry. The paper was then suspended in a solvent

mixture of lutidine, water, and cyclohexanone (42-48-10). The use of cyclohexanone was based upon its strong solubilizing properties of the free porphyrins. The chromatogram was placed in the refrigerator and allowed to run for approximately 18 hours at 2°C. After development the wet paper was viewed under an ultraviolet light and the free porphyrin could be visualized on the paper. This technique separated the mixture of free porphyrins into a number of separate spots. The porphyrins migrated in the order of uroporphyrin, coproporphyrin, and protoporphyrin with increasing mobilities. From the chromatogram it was obvious that a number of red fluorescent areas other than coproporphyrin and uroporphyrin were obtained. This is in agreement with the results obtained by isolating the methyl esters from the silicagel columns. Attempts were made to adjust the composition of the solvent mixture by varying the percentage of lutidine and cyclohexanone in order to resolve the various isomers. Satisfactory results were not obtained although slight separation was seen.

Authentic samples of uroporphyrin, coproporphyrin, and protoporphyrin were placed on 25 cm. square papers along with a mixture of the porphyrins previously isolated. After development of the chromatogram, as previously described, spots corresponding to uroporphyrin and coproporphyrin were seen; in addition two fluorescent areas between uroporphyrin and coproporphyrin were visualized and an area between coproporphyrin and protoporphyrin was visualized. From these results as shown in Figure 7, it would appear that porphyrins containing three, five, and seven carboxyl groups were isolated from the urine in the squirrel.

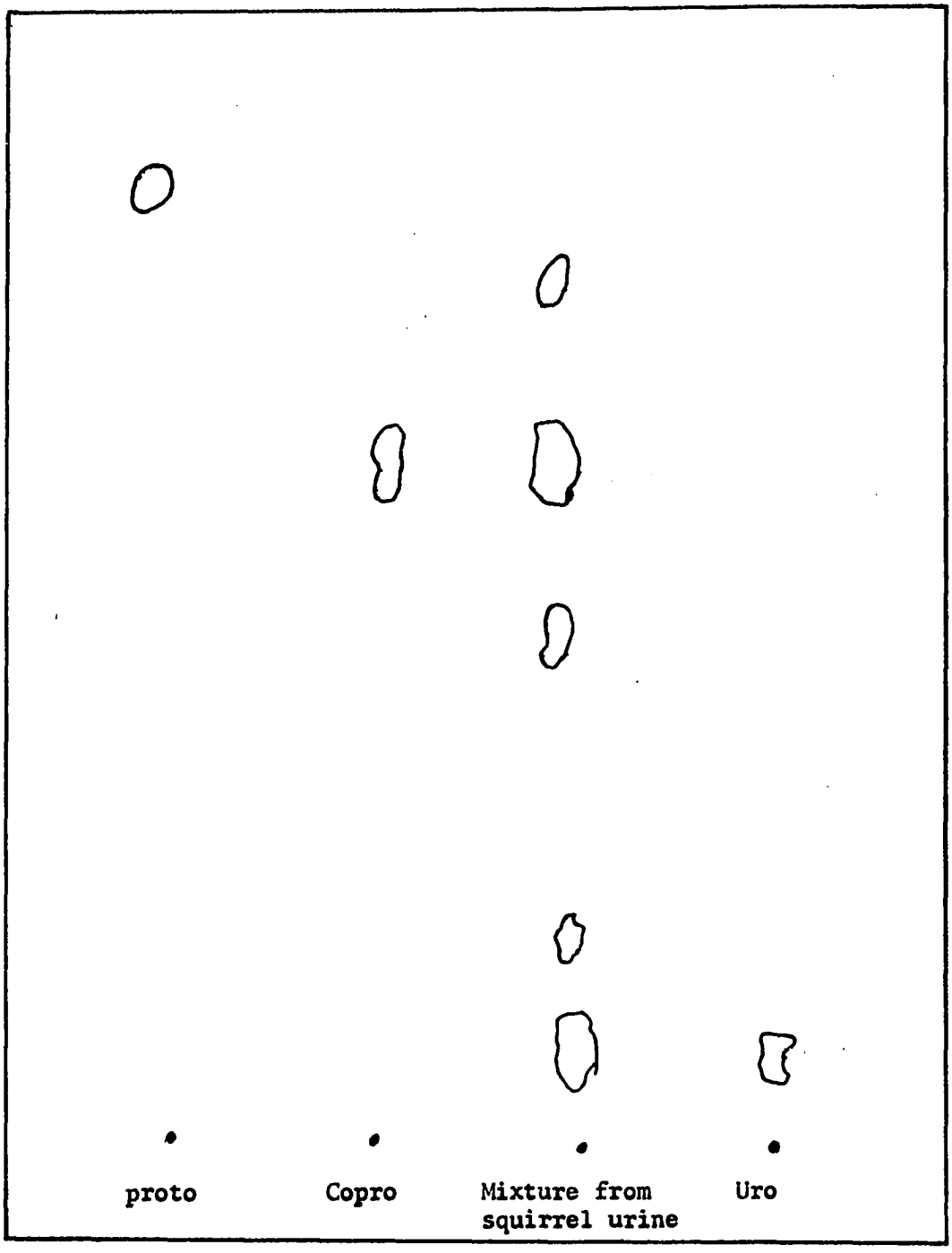


Fig. 7. Ascending paper chromatography of free porphyrins on Whatman Number 1 Paper at 2°. Solvent 2,6-lutidine, water, cyclohexanone 42:48:10.

The samples of free porphyrins utilized in this study were obtained by hydrolyzing the porphyrin methyl esters with hydrochloric acid overnight, extracting the porphyrins, evaporating to dryness, and dissolving the material in 10 normal ammonium hydroxide. This treatment could possibly lead to the production of artifacts, therefore it was felt in order to determine if the fluorescent areas which did not correspond to uroporphyrin or coproporphyrin were being produced by the hydrolysis that chromatograms of the methyl esters would be studied. The results of this study are given in the next section.

Paper Chromatography of Porphyrin Methyl Esters

The combined methods of Chu (9) and Falk (18) were used to chromatograph the methyl esters of porphyrins isolated from the squirrel urine. This technique consisted of two dimensional chromatography run on 19 cm. squared of Whatman No. 1 filter paper. The sample was placed in the lower right hand corner approximately 1 inch from either margin of the paper. The first solvent system used was a mixture of odorless kerosene and chloroform (6.0 - 3.9). The chromatogram was developed until the solvent front had ascended approximately 15 cm. Chloroform was placed at the bottom of the chamber during this run in order to saturate the atmosphere. The entire run was performed at room temperature. The paper was removed from the chamber and dryed for four minutes at 105-110°C. The paper was once again returned to the chromatography chamber and placed in a solvent mixture of kerosene and normal porpanol (7.5 - 1.5). Kerosene was placed in the bottom of the chamber to saturate the atmosphere. The solvent was allowed to migrate to approximately the same position as the first solvent. The paper was then dryed

for four minutes at 105-110°C. The paper was turned 90° and placed in a third solvent system of kerosene and p-dioxane. The solvent was allowed to migrate to the top of the paper. After the third development the porphyrin esters were located on the paper by their fluorescence under an ultraviolet lamp.

A control chromatogram of the isomers of uroporphyrin and coproporphyrin, protoporphyrin and mesoporphyrin was run under similar conditions in order to obtain a map of the separation obtained by the above system. Each isomer and porphyrin was run individually in order to obtain the relative mobility and a composite chromatogram of the results was prepared. A mixture of coproporphyrin I, coproporphyrin III, uroporphyrin I, uroporphyrin III, protoporphyrin, and mesoporphyrin was prepared and chromatographed under the same conditions. The results of these studies show that the relative mobilities of the porphyrin methyl esters were uroporphyrin, coproporphyrin, protoporphyrin, and mesoporphyrin. The No. 3 isomer of coproporphyrin and uroporphyrin had a greater mobility than that of the No. 1 isomer.

Chromatograms were prepared for the porphyrin methyl esters isolated from the squirrel's urine and still in a relatively crude state before any separation by silica gel chromatography, and chromatographed in the system described. Simultaneous control chromatograms of the mixture of authentic porphyrin methyl esters was always run at the same time. After development of the unknown mixture of crude porphyrin methyl esters the spots were visualized under ultraviolet light and showed areas corresponding to uroporphyrins I and III, coproporphyrin I and III, and other fluorescent zones intermediate between the uroporphyrin and

coproporphyrin areas and between the coproporphyrin and protoporphyrin areas as shown in Figure 8. These areas produced a typical red-orange porphyrin fluorescence on paper and were tentatively identified as porphyrins containg three, five, six, or seven carboxyl groups.

Fractions rich in coproporphyrin which had been isolated from the silica gel columns were chromatographed and the results showed that these fractions contain material with chromatographic mobilities essentially identical to those of pure coproporphyrin I and coproporphyrin III. Likewise fractions of methyl esters obtained from silica gel columns that were rich in uroporphyrin when chromatographed showed identical mobilities to that of authentic uroporphyrin I and III. Chromatography of fractions obtained from a large amount of squirrel urine by silica gel chromatography, similar to that in Table 3, and showing a red fluorescence was performed and various of the minor fractions showed spots with mobilities dissimilar to those of either coproporphyrin or uroporphyrin. This further substantiated the belief that the smaller fractions obtained from large samples of urine were discrete entities. The results also agreed with those obtained from a mixture of the crude fractions before silica gel chromatography and indicated that porphyrins of three, five, six, or seven carboxyl groups were being excreted and isolated from the urine of the squirrel.

Determination of Substances Reacting with Erlich's Reagent

In acute intermittent porphyria it is known that porphobilinogen is excreted in the urine in large quantities. This substance is a substituted pyrrole compound which will react with Ehrlich's reagent

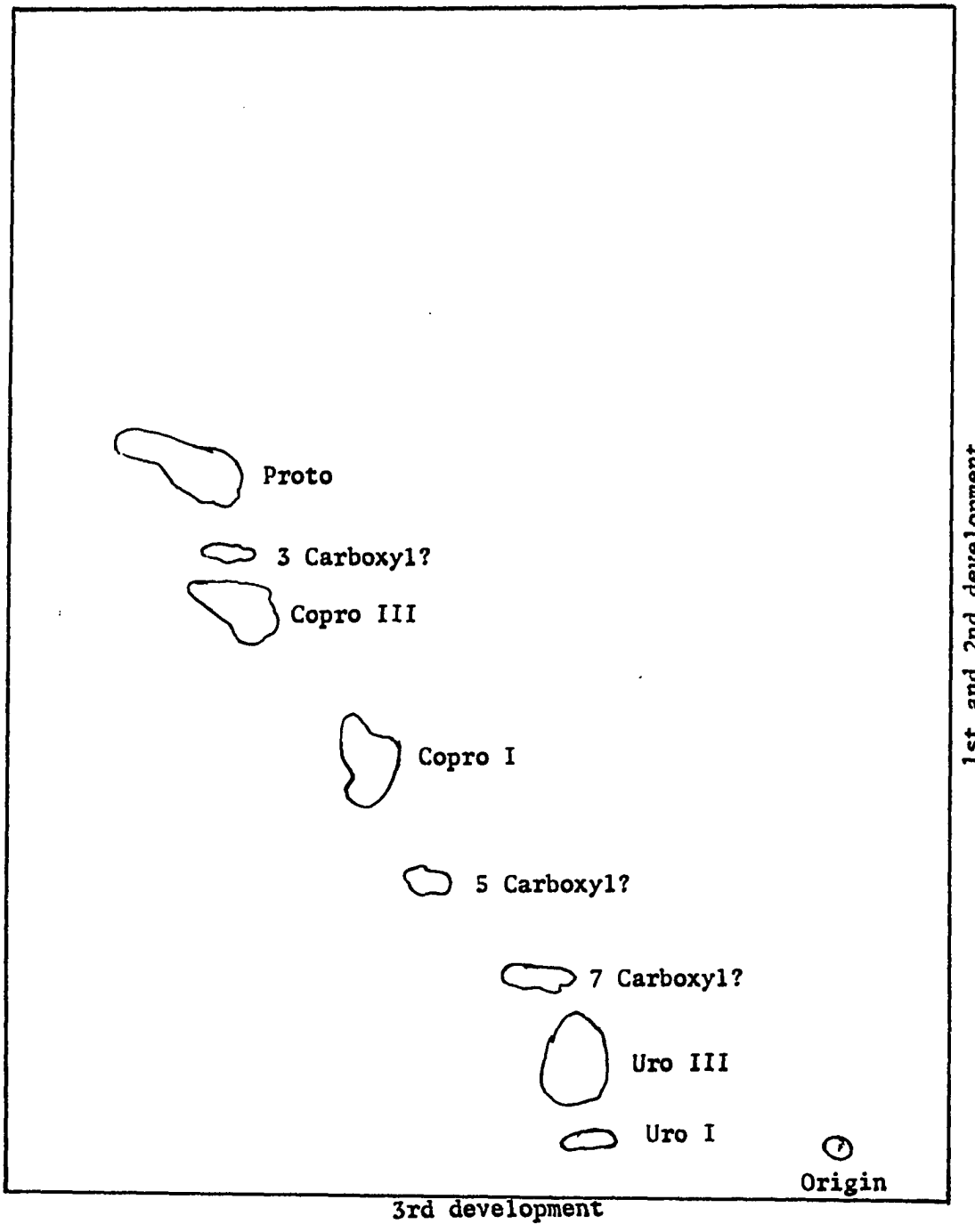


Fig. 8. Ascending paper chromatography of porphyrin methyl esters from squirrel urine. Whatman Number 1 paper. 1st solvent kerosene and chloroform 6:3.9; 2nd solvent kerosene and n-propanol 7.5:1.5; 3rd solvent kerosene and p-dioxane 8:3. Temperature 26°. Protoporphyrin added as a marker.

in an acid medium. To determine if the urine from the squirrel which contained a number of porphyrins possessed any Ehrlich's reacting substances the urine was tested with a modified Watson Schwartz reagent. This reagent consists of p-dimethylaminobenzaldehyde dissolved in hydrochloric acid. Urines containing pyrrole with a free alpha position when reacted with the Ehrlich's reagent will form a cherry red color. If porphobilinogen is present it may be differentiated from urobilinogen which also reacts with the Ehrlich's reagent by adding an equal volume of saturated sodium acetate to the Ehrlich's reaction mixture and then extracting the resulting solution with chloroform. If porphobilinogen is present, the aqueous layer will retain a cherry red color and any urobilinogen will be extracted into the chloroform layer. When this test was applied to freshly voided squirrel urine results were obtained which showed that porphobilinogen was being excreted in the urine of the squirrel. To further confirm the presence of porphobilinogen in the urine of the squirrel the method of Mauzerall and Granick (43) was applied to samples of freshly voided urine. This method is based upon the preferential removal of porphobilinogen from urine at a slightly acid pH by Dowex 2 resin in the acetate form. Columns of Dowex 2 resin were prepared 2 cm. in height and 1.0 cm. in diameter. A 2 ml. sample was applied to the column and the column was allowed to drain completely dry. The column was then washed with 2 ml. portions of water. The porphobilinogen was eluded from the column by adding 2 ml. of 1 normal acetic acid followed by 2 ml. of .2 normal acetic acid. The eluate was collected in a 10 ml. volumetric flask and diluted to 10 ml. with water. A 2 ml. sample of the resulting solution

was reacted with a modified Ehrlich's reagent and showed a faint cherry red color. The modified Ehrlich's reagent used consisted of 0.5 gm. of p-dimethylaminobenzaldehyde dissolved in 15 ml. of glacial acetic acid to which 4 ml. of 70% perchloric acid was added. The resulting solution was diluted to 25 ml.. This reagent is more sensative than the Ehrlich's reagent made in hydrochloric acid.

Additional evidence was obtained that the Ehrlich's positive material obtained from the Dowex 2 column was porphobilinogen was obtained by passing a larger volume of freshly voided urine through columns containing 3 to 5 times the amount of Dowex 2 resin as used for a 2 ml. sample of urine. The eluate was collected in fractions and those having the highest concentration of Ehrlich's positive material were used to identify the subject on paper chromatograms. A portion of the unreacted eluate was spotted on Whatman No. 1 paper and placed in a solvent system of butanol-acetic acid and water. The solvent system was prepared by mixing four volumes of N-butanol, and volume of glacial acetic acid, and five volumes of water. This solution was thoroughly mixed and after complete separation the upper phase was withdrawn and used as a solvent system for chromatography. The chromatogram was developed until the solvent front had reached the top of the paper and then removed from the chamber and air dryed. After drying the paper was carefully sprayed with the previously described modified Ehrlich's reagent. Only one pink spot was attained and this had a relative mobility of 0.5 which corresponds with that reported by Mauzerall and Granick (of 0.5 for a sample of pure porphobilinogen.

Further identification of the substance eluted from the

Dowex 2 column was obtained by reacting a portion of the eluate with the modified Ehrlich's reagent and running the continuous absorption spectrum of the resulting product on the recording spectrophotometer. The resulting absorption curve showed the porphobilinogen Ehrlich's reaction product to have a maximum at 555 mμ. This corresponds with data published by Mauzerall and Granick (43).

From the results obtained by the various methods just described it can be concluded that freshly voided urine of the squirrel contains porphobilinogen. Only one spot which would react with Ehrlich's reagent was obtained on paper chromatography, however, it is difficult to visualize Ehrlich's positive pyrroles on paper chromatograms because after reaction with the Ehrlich's reagent the resulting product rapidly fades and can no longer be visualized after about 30 seconds.

Delta-aminolevulinic acid is another precursor in the biosynthetic pathway of porphyrin metabolism that is excreted in acute intermittent porphyria. To ascertain if this substance were being excreted in the urine of a squirrel samples of urine were passed through a Dowex 2 column which was then washed with two 2 ml. portions of water. The urine and water washings were combined and placed on a column of Dowex 50 resin in the hydrogenion form and allowed to drain dry. The column was then washed with large amounts of water until any urea present was removed. The absence of urea in the washings was checked on a spot plate by reacting the washings with Ehrlich's reagent. Urea and Ehrlich's reagent give a lemon yellow color. After all urea had been removed from the column the column was washed with 3 ml. of 0.5 molar

sodium acetate which was discarded. The delta-aminolevulinic acid was eluted from the column with 7 ml. of 0.5 molar sodium acetate. The eluate was collected in a 10 ml. volumetric flask and 0.2 ml. of 2,4-pentanedione was added to the solution. The solution was diluted to 10 ml. with a pH of 4.6 acetate buffer. The flask was stoppered and placed in a boiling water bath for 10 minutes and then cooled to room temperature. A 2 ml. sample of the resulting solution was reacted with the modified Ehrlich's reagent. A typical cherry pink color resulted indicating that a substance was being excreted in the urine which would condense with 2,4-pentanedione to form a pyrrole with a free alpha position thus capable of reacting with Ehrlich's reagent.

The solution obtained by reacting 2,4-pentanedione with the eluate from the column was spotted on Whatman No. 1 paper and chromatographed in the same solvent system as used for porphobilinogen. At development of the chromatogram the paper was again sprayed with a modified Ehrlich's reagent and a faint pink spot was obtained with an Rf value of 0.9. This is the same as reported for the pyrrole resulting from the condensation of delta-aminolevulinic acid with 2,4-pentanedione by Mauzerall and Granick (43). In addition to the spot obtained which corresponded to that resulting from delta-aminolevulinic acid an additional spot which was Ehrlich's positive was also seen on the chromatogram. A similar series of reactions is utilized to convert glucosamine to a pyrrole which then reacts with Ehrlich's reagent. To rule out the possibility that glucosamine was responsible for the second spot observed, a sample of pure glucosamine was dissolved in water, the pH

adjusted to six and treated similar to a sample of urine which was passed through the columns. No condensation with 2,4-pentanedione at pH 4.6 occurred as the resulting solution would not react with the modified Ehrlich's reagent. This indicated that glucosamine was not the substance responsible for the second spot observed. It was felt that a number of amino acids might be responsible for the second Ehrlich's positive spot observed on the paper chromatograms and therefore a sample of the unreacted eluate obtained from the Dowex 50 column was placed in the lower right corner of a 19 cm. square of Whatman No. 1 filter paper. The chromatogram was developed in the first dimension in a solvent system of butanol acetic acid and water and then turned 90% and developed in a second solvent system of phenol and water. The paper chromatogram was allowed to air dry for 48 hours and then sprayed with an ninhydrin solution. The paper was allowed to air dry for five minutes and then placed in an oven at 60°C. A number of ninhydrin positive spots resulted thus indicating that the eluate of the Dowex 50 column contained a number of amino acids.

A larger amount of urine consisting of a freshly voided and mixed 20 ml. sample of urine was passed through the Dowex 2 and 50 columns and collected. The eluate from the Dowex 50 column was not reacted with 2,4-pentanedione but was used to further identify the ninhydrin positive spots observed previously. A portion of the eluate was spotted on chromatography paper and the chromatograms were developed in the solvent systems previously described. After spraying with ninhydrin a number of spots were visualized. These corresponded to an area which normally contains leucine, isoleucine, and phenylalanine. A second area corresponds to that

of valine or methionine. A third area corresponds to that of tyrosine. The fourth ninhydrin positive area corresponds to that of glycine and a fifth which was initially yellow in color corresponds to that of delta-aminolevulinic acid.

Each of the above described amino acids was dissolved in water and the resulting solutions were treated in the same manner as an eluate from Dowex 50 column. On reaction with 2,4-pentanedione at a pH of 4.6 the resulting solution remained clear. Portions of the reaction mixture of each of the individual amino acids were taken and to them was added a modified Ehrlich's reagent. None of the above mentioned amino acids with the exception of delta-aminolevulinic acid gave an Ehrlich's positive color. From these results it was believed that none of the amino acids identified on the paper chromatograms were responsible for the Ehrlich's positive reaction.

Upon passing an aqueous solution of pure delta-aminolevulinic acid through columns of Dowex 2 and Dowex 50 resin and subsequently reacting this with 2,4-pentanedione it was noted that the resulting solution was clear. In contrast many samples of squirrel urine treated in the same manner upon heating with 2,4-pentanedione showed a yellow greenish color. The resulting yellow greenish solution when reacted with modified Ehrlich's reagent gave a color which was very similar to that given by pyrroles plus Ehrlich's reagent. However, when the continuous absorption spectrum of the Ehrlich's product with the yellow greenish solution was studied by running the continuous absorption spectrum it was found that a broad peak ranging between 510-535 mμ. was obtained. The usual maximum absorption for pyrroles plus Ehrlich's reagent will

show a peak in the 550 mμ range.

Attempts were made to fractionate the material obtained from the Dowex 50 columns that would react with Ehrlich's reagent by passing up to 350 ml. of urine through the columns. The eluate from the Dowex 50 columns was collected on a fraction collector and fractions of 10 ml. were collected. The collection was continued until an aliquot of one of the 10 ml. fractions no longer gave an Ehrlich's positive reaction after condensation with 2,4-pentanedione. When samples as large as 200 ml. of urine were processed in this manner 30-40 10 ml. fractions were normally obtained which would produce a yellow-greenish color upon reaction with 2,4-pentanedione. The resulting solutions when reacted with Ehrlich's reagent gave colors ranging from a purplish-red to a orange-pink color. It was not possible to ascertain if the color intensity were due to a concentration affect or to separate compounds. The absorption spectrum of each of the fractions was determined and rather broad peaks were obtained thus indicating a probable mixture of compounds. In order to obtain fractions containing single compounds, large columns of Dowex 50 resin were used and the flow rate of the eluate was controlled so that more discrete fractions could be obtained. When as many as 75 separate fractions were collected, it was possible by controlling the flow rate to obtain as many as 50-60 fractions which reacted with 2,4-pentanedione to give a slight yellow-greenish color and subsequently reacted to give an Ehrlich's positive color which ranged from a faint peach color to a typical purple color of delta-aminolevulinic acid. Figure 9 shows the elution pattern from the Dowex 50 column.

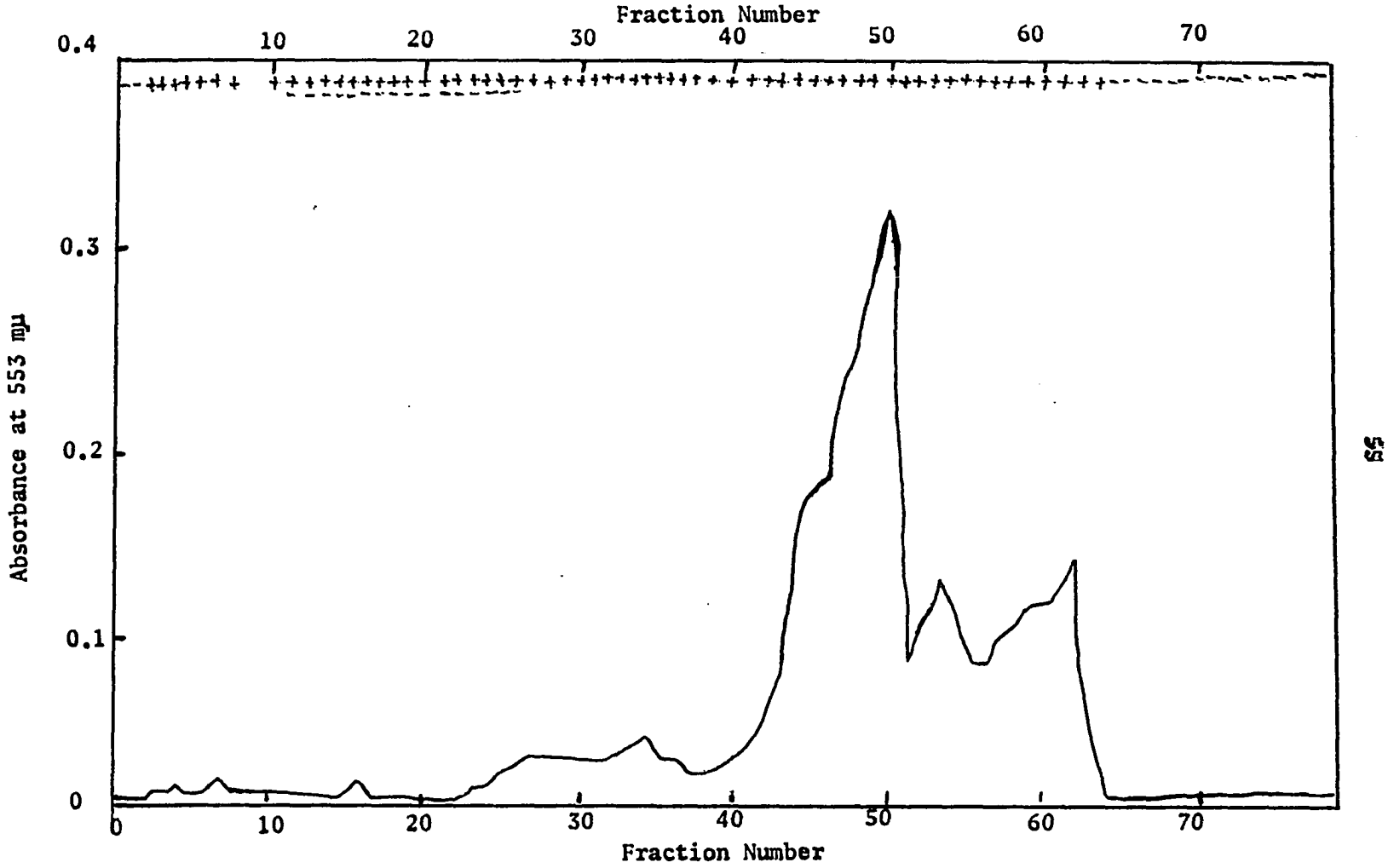


Fig. 9. Elution pattern from Dowex 50 column. The solid line represents the concentration of Ehrlich's reactants, the + and - represent the degree of color formation of eluate with 2-4 pentanedione.

A fraction was chosen which showed an intense yellow color after reaction with 2,4-pentanedione. An aliquot of this fraction was taken and efforts made to remove the yellow color from the solution. The pH was adjusted to 7.0 with dilute sodium hydroxide. This was then extracted with several portions of chloroform. The chloroform extract contained all of the yellow color from the original solution. The aqueous layer when reacted with Ehrlich's reagent gave no reaction. The chloroform layer was evaporated to dryness with an amber colored oil resulting. The amber oil was shaken with distilled water and the water extract became yellow. The water extract when reacted with Ehrlich's reagent gave no reaction. The pH of a portion of the water extract was lowered to 1 with 6 normal hydrochloric acid. A portion of this solution was also negative when reacted with Ehrlich's reagent. The acid solution was allowed to stand for several hours and placed in a refrigerator, however, no perceptible crystallization occurred. The solution was next heated at 80°C for several minutes. As the heating progressed the yellow solution changed from a bright yellow color to a nearly colorless solution. A portion of this solution was reacted with Ehrlich's reagent and gave an extremely intense purple color typical of that obtained from the reaction of Ehrlich's reagent with a pyrrole. A portion of the water extract whose pH had been adjusted to 1 with hydrochloric acid was once again reacted with Ehrlich's reagent and after standing for several hours the color of the solution became less intense and after further standing a purple color developed. This would indicate that the presence of strong acid was responsible for the change from a yellow color to that of a clear solution. A further portion of the water

extract was reacted with Ehrlich's reagent and after standing for several hours the same results were obtained as when the pH of the water extract had been lowered by the addition of hydrochloric acid. These results seem to indicate that the alpha position of the pyrrole was probably substituted by a labile group that was being hydrolized in the presence of an acid and the process was further catalyzed by heating. The Ehrlich's reagent is made up in glacial acetic acid and perchloric acid with an extremely low final pH. It was felt that this acid reagent was having the same effect on the water extract as was hydrochloric acid. (Figure 10).

Approximately 200 ml. of urine was passed through the Dowex 2 and Dowex 50 columns and the material which was retained on the Dowex 50 column was once again eluted and collected in fractions. One ml. aliquots of each fraction were reacted with 2,4-pentanedione and all fractions which showed a yellow or yellow-greenish color were pooled. The pooled solution was mixed with 1 ml. of 2,4-pentanedione and 50 ml. of the pH 4.6 buffer and heated for 10 minutes. An intense yellow color resulted. This entire solution was adjusted to a pH of 7 and extracted with copious volumes of chloroform. The chloroform extract was an extremely bright yellow color and after evaporation of the chloroform on a turning evaporator a small amount of an amber oil was once again obtained. This oily solution was repeatedly extracted with water until the water extracts remained colorless. The water extract was divided into several portions and set in the refrigerator until further work could be done on these fractions.

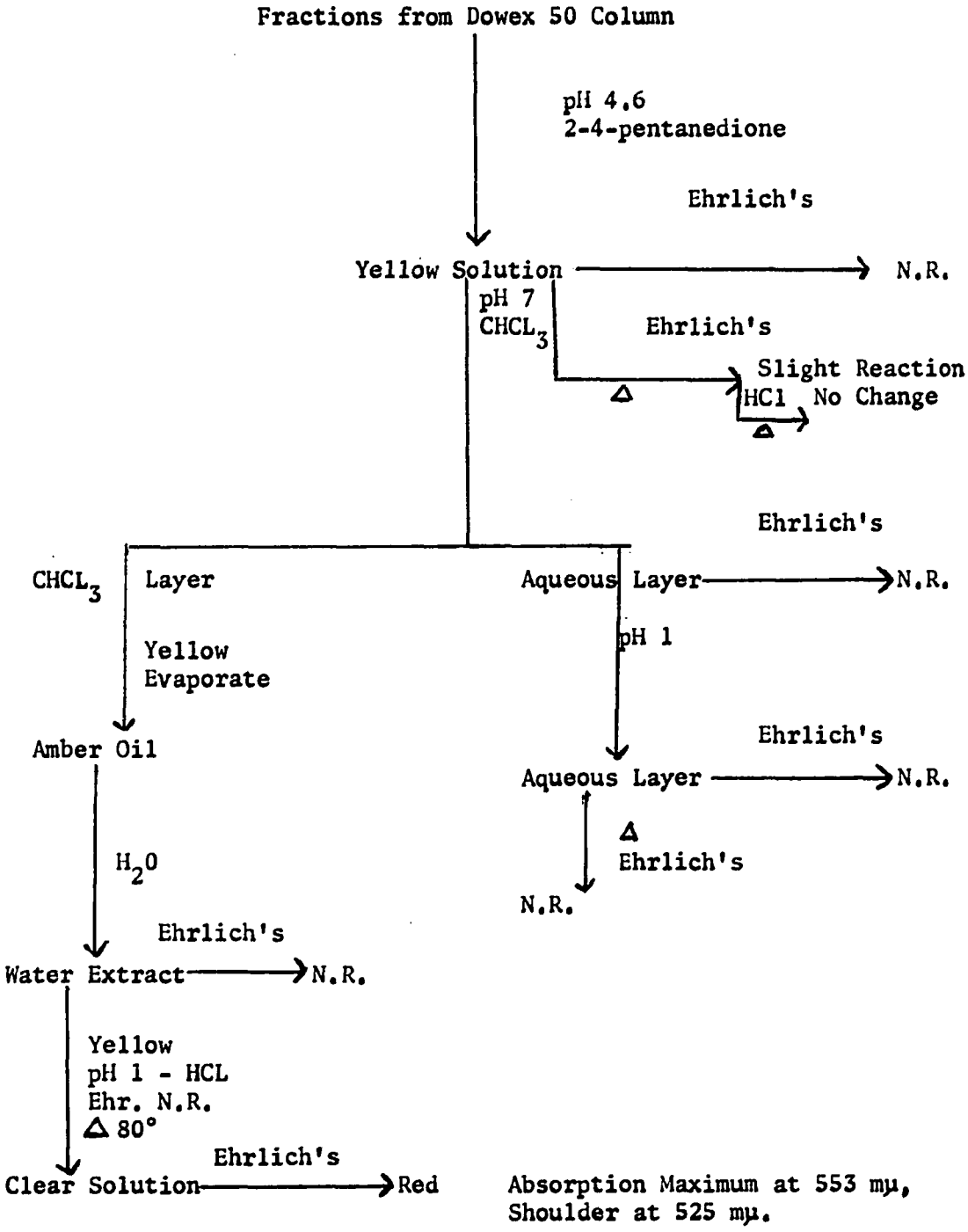


Fig. 10. Flow sheet of steps used to convert the fractions from the Dowex 50 column which gave a yellow product when reacted with 2-4-pentanedione, to a compound which reacted with modified Ehrlich's reagent to give the same absorption spectrum as 2-methyl-3-acetyl-4(3-propionic acid) pyrrole.

The water extract was spotted on Whatman No. 1 filter paper and chromatographed in a system of butanol-acetic acid-water. After four and one-half hours at room temperature, the solvent had migrated some 15 cm. past the origan. The water extract showed several spots after treating the paper with hydrochloric acid and heat and then sprayed with Ehrlich's reagent. None of the spots which appeared corresponded to that of the pyrrole resulting from the condensation of delta-aminolevulinic acid with 2,4-pentanedione. A second chromatogram which was run simultaneously but not sprayed with hydrochloric acid or heated showed one spot which was positive when the chromatogram sprayed with Ehrlich's reagent. This would indicate that the water extract contained a pyrrole which was in such a form that it was able to react with Ehrlich's reagent without further treatment and that the extract also contained a compound which was substituted in the alpha position thus blocked any reaction with para-dimethylaminobenzaldehyde until it was treated with acid and heated.

When a portion of the water extract was reacted with Ehrlich's reagent, a very slow reaction took place and after approximately 2 hours a purplish color resulted. When the continuous absorption spectrum of this solution was obtained, it was found that a rather broad maximum was obtained in the region of 520-545 (Figure 11). When the same water extract was treated with hydrochloric acid and heat and then reacted with Ehrlich's reagent a very prompt purple color developed which was quite stable The absorption spectrum of this solution showed a sharp peak at 553 mμ. (Figure 12).

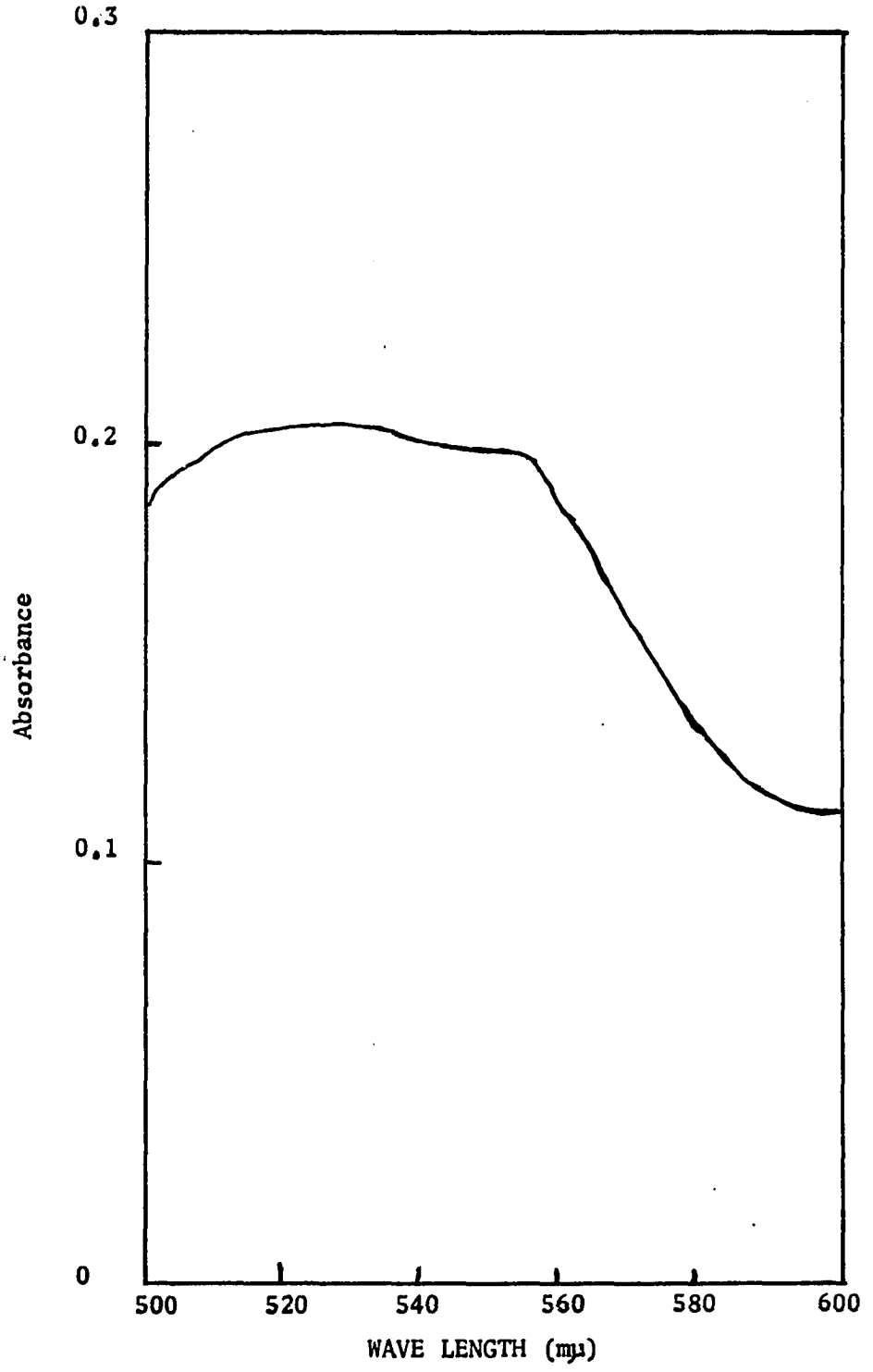


Fig. 11. Absorption spectrum of water extract, before being heated at pH 1, reacted with modified Ehrlich's reagent.

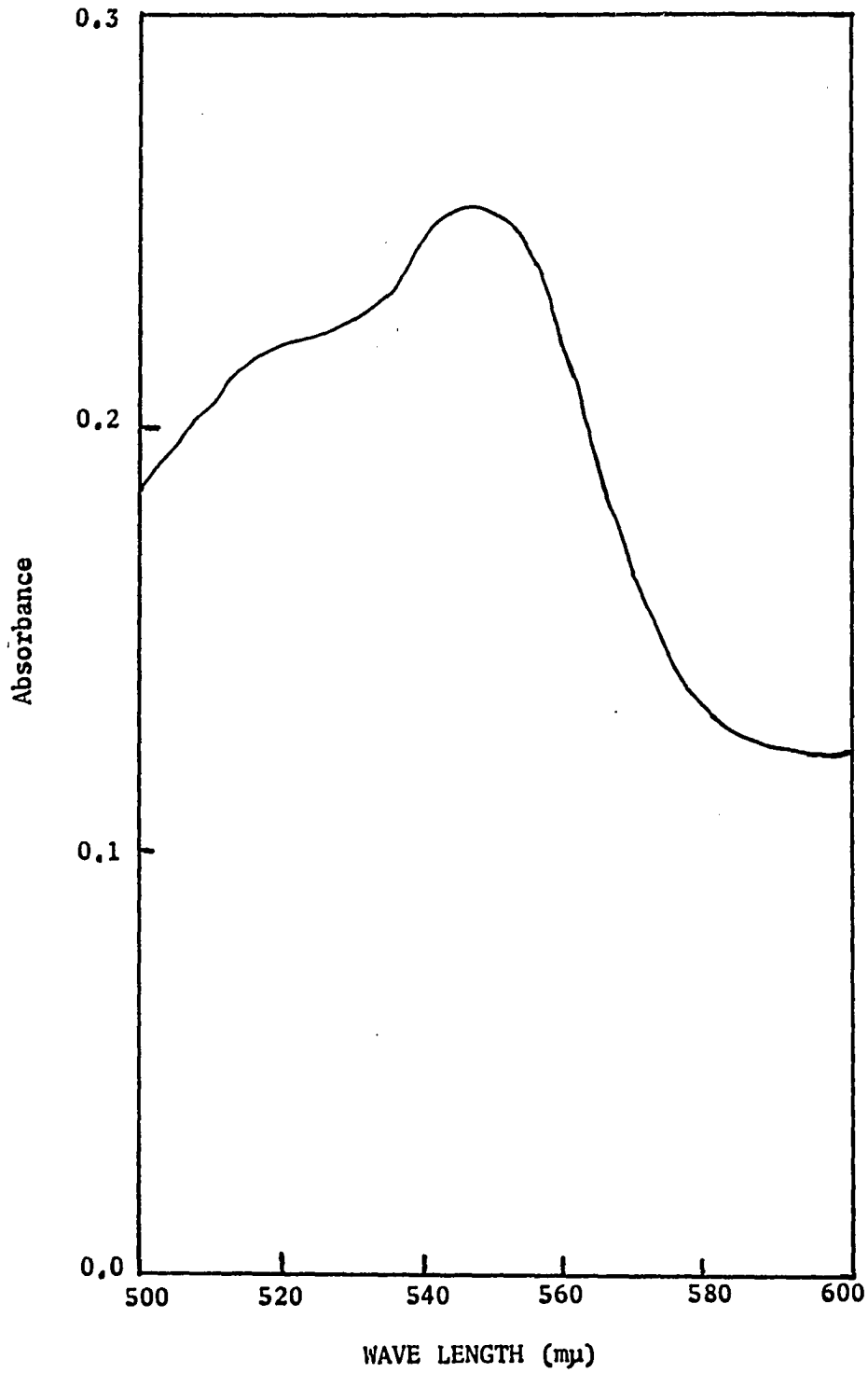


Fig. 12. Absorption spectrum of water extract heated at pH 1 and reacted with modified Ehrlich's reagent.

Findings in Patients with Symptoms of Acute Intermittent Porphyria

In an effort to see if humans, suffering from symptoms of acute intermittent porphyria, excreted substances similar to those found in the urine of the fox squirrel those individuals with a past history and diagnosis of acute intermittent porphyria were subjected to study. Other criteria used for classifying patients as suffering from acute intermittent porphyria were those of abdominal pain, paralysis, and mental aberrations. Urinary findings on these individuals included the excretions of abnormal quantities of porphyrins and either porphobilinogen and/or delta-aminolevulinic acid. These patients were initially classified as suffering from acute intermittent porphyria if the subjective clinical findings were those just described and if the urine was shown to contain porphobilinogen by the Watson-Schwartz method (80). On re-evaluation and study of these patients the urine of each patient was collected for a twenty-four hour period, preserved in a refrigerator in an amber bottle containing sodium carbonate. The porphyrin level was determined utilizing a Turner Model III fluorometer by the method of Talman (66). The fluorometer was equipped with a high sensitivity cuvette holder. The primary filter was a 405 and a 25 was used as the secondary filter. Porphobilinogen and delta-aminolevulinic acid was determined by the method of Mauzerall and Granick (43). A number of the individuals studied had been hospitalized repeatedly with acute attacks over a period of several years and others were studied during the initial episode of acute intermittent porphyria. Those with a previous history of acute intermittent porphyria were maintained on large quantities of Thorazine for the symptomatic relief of the symptoms. When the urine of these

individuals was collected for study they had been removed from all medication for several days prior to the initial collection of urine.

Because porphyrin excretion may be elevated in conditions other than porphyria it was necessary to eliminate the possibility that the patients were suffering from other conditions at the time of study. As reported by Schwartz (59) the level of urinary porphyrins may increase markedly in such conditions as lead poisoning, poliomyelitis, various liver diseases, a number of types of anemia, acute alcoholism with damage to the liver, and in Hodgkin's disease. Appropriate studies were performed to rule out these conditions before the patient was judged to be suitable for the study. Schwartz, Zieve, and Watson (61), reported that in acute intermittent porphyria as much as fifty per cent of the total porphyrin excreted by individuals suffering from acute intermittent porphyria was excreted in the form of the colorless porphyrinogens. Because of this observation the free porphyrins which were excreted were measured and the total porphyrins were measured after oxidation of the urine sample with dilute iodine solution. The method for separating uroporphyrins from coproporphyrins was based on the fact that at a pH of approximately 4.8 the coproporphyrins may be extracted with ethyl acetate and the uroporphyrins remain in the aqueous phase. The aqueous phase was then treated with a small amount of aluminum oxide and the uroporphyrins were subsequently removed from the aluminia by suspending the aluminia in 1.5 normal hydrochloric acid. The uroporphyrins are removed from the aluminia by this treatment. The aluminia was repeatedly extracted with the hydrochloric acid until the eluate showed essentially no fluorescence over that of the blank when measured in the

fluorometer. In urines containing a large amount of uroporphyrin it was necessary to extract five to ten times yielding a total volume of approximately 50 mls. of hydrochloric acid. The combined eluates were then diluted to 50 ml. with 1.5 normal hydrochloric acid and mixed thoroughly and the fluorescence of the resulting solution measured. The uroporphyrin precursors including uroporphyrinogen were converted to uroporphyrin by treating the sample with glacial acetic acid at a pH of 5 and heating in a boiling water bath for thirty minutes. During this heating process the contents of the flask were protected from light by covering the flask with aluminum foil. By the methods just described the normal daily excretion of total coproporphyrins is between 75 and 300 mcg. per day and that of total uroporphyrins to 50 mcg. per day. Those patients studied showed a variable excretion with most exceeding the upper limits of normal porphyrin excretion. Some of the individuals excreted porphyrins in quantities that were not abnormal on certain occasions and other times their porphyrin excretion exceeded that of the normal by as much as three to four times. Because of this observation it was felt that it was necessary to analyze each individual specimen that was voided during a twenty-four hour period in order to ascertain if the excretion of porphyrins in the urine was an intermittent process. When this was done as many as five to six individual urine samples were obtained during a 24-hour period. Based upon a comparison of the volume of urine excreted it was found that a single voiding often contained a quantity of porphyrin exceeding that of the normal twenty-four output. On other occasions a single voiding would contain extremely low amounts of porphyrin. After studying several patients it was obvious that the

excretion of porphyrins in the urine was not at a constant level and therefore made it necessary to study aliquots of pooled twenty-four-hour samples. When the level of urinary porphyrins was in excess of that found in normal individuals the urine from the patient was collected over a period of several days, pooled and the porphyrin was isolated, esterfied, and purified partially by the methods that were utilized in studying the urine of a squirrel. Small columns of silica gel were prepared and the porphyrins were further purified by this manner. The fractions that were isolated from the silica gel columns were chromatographed on Whatman #1 paper and it was found that the isomer distribution was similar to that found in the fox squirrel. It should be noted that the level of porphyrin excreted was much lower than that in the fox squirrel and the smaller amounts of the porphyrins containing odd number carboxyl groups found in the squirrel were not detected in the urine of the patients suffering from acute intermittent porphyria. It was routinely found that the total urinary excretion of porphyrins was made up of approximately 50 per cent preformed porphyrins and 50 per cent porphyrins that were being excreted as porphyrinogens. This was determined by isolating the preformed porphyrins from an aliquot of urine and on a second aliquot of the same urine specimen the total porphyrins were measured after converting the porphyrinogens to porphyrins by appropriate methods. The ratio of preformed porphyrins to total porphyrins varied from individual to individual but the average of 38 patients was found to be approximately 50 per cent.

Aliquots of freshly voided urine were taken before addition of the specimen to the 24-hour collection bottle and subjected to analysis

for porphobilinogen and delta-aminolevulinic acid utilizing the method of the ion exchange resins. The finding of delta-aminolevulinic acid like substances in the urine specimens were analyzed for a period of from seven days to 35 days consecutively were found to contain essentially no delta-aminolevulinic acid at varying intervals. However, on approximately 75 per cent of the consecutive days the eluate from the Dowex 50 column gave a positive reaction with the modified Ehrlich's reagent. In several of the patients the eluate after condensation with 2,4-pentanedione was noted to have the yellow-green color that was a consistent finding in the fox squirrels. The majority of the patients excreted urines which did not contain a substance when reacted with 2-4 pentanedione after passage through ion exchange resins that would give this greenish-yellow color. On those individuals whose urine contained no substance which by appropriate treatment showed a greenish-yellow color, it was found that on paper chromatography of the eluate from the Dowex 50 column that only delta-aminolevulinic acid was detected by spraying the chromatogram with ninhydrin. These specimens which after condensation with 2,4-pentanedione yielded a water clear solution that could be shown to contain more than one Ehrlich's positive compound, however. Gibson, Laver, and Neuberger (23) showed in 1958 that other amino ketones when reacted with 2,4-pentanedione would give a positive reaction with Ehrlich's reagent. They also showed that by adjusting the pH of the resulting solution to 8 or more and extracting with ethyl ether the pyrrole resulting from amino acetone passed into the ether phase and that the pyrrole resulting from delta-aminolevulinic acid remained in the aqueous phase. Likewise other amino ketones that were converted to

pyrroles remained in the aqueous phase. By adjusting the pH of the pyrrole containing solution obtained after treatment of the Dowex 50 eluate to a pH of 8 with sodium hydroxide and extracting with ethyl ether it was found that in a number of specimens the aqueous phase persisted in giving a positive reaction with Ehrlich's reagent and that the ether phase after evaporation to dryness would also give an Ehrlich's reaction. In those individuals it appeared that they excreted both delta-aminolevulinic acid and a substance presumed to be amino acetone that was extracted by ether at an alkaline pH. Large samples of urine were passed through the Dowex 2 and 50 columns and subsequently condensed with 2,4-pentanedione to form a pyrrole. After removal of excess 2,4-pentanedione the pyrrole containing solution was spotted on Whatman #1 filter paper and chromatographed. The solvent system was that of butanol, acetic acid, and water and the analysis was run at room temperature. The known pyrrole from the reaction of delta-aminolevulinic acid and 2,4-pentanedione was run simultaneously. After several hours the solvent system would move approximately 15 cm. The chromatogram was removed from the chromatographic chamber and allowed to air dry then sprayed with the modified Ehrlich's reagent. In those specimens which had a compound extractable by ether at alkaline pH's it was found that two Ehrlich's positive spots were obtained. One of these had an R_f value corresponding to that of a pyrrole from delta-aminolevulinic acid. The other had a mobility of much less than that of the known pyrrole. Urine samples from normal individuals were collected for a period of 24 hours and preserved in the usual manner. Aliquots of these specimens were subjected to analysis by the technique of the ion exchange resins in order to find if a compound

was being excreted that had similar chromatographic properties to that extracted by ether from the Dowex 50 columns of patients suffering from acute intermittent porphyria. In analyzing a large number of these urines, it was not possible to detect any Ehrlich's positive substances in the eluate of the Dowex 50 column. Therefore, it appears that the substance which is extracted by ether in an alkaline pH from the reaction mixture of Dowex 50 eluate and 2,4-pentanedione is a compound of abnormal nature.

Several individuals were found whose urine upon heating with 2,4-pentanedione buffered at pH of 4.6 showed urines that turned the greenish-yellow color so typical of that seen in the urine of a fox squirrel. In the specimens from the porphyric individuals this color was extremely deep. The patients studied in this group ranged in age from 8 years to 50 years. All of these individuals had had previous episodes similar to those seen in acute intermittent porphyria. Over the course of 12 months the urine samples were collected seven times from one individual at intervals varying from 3 weeks to 2 months. On one occasion this individual was studied for 17 consecutive days. Each 24-hour collection revealed the same findings. The urine appeared normal in nature but contained in excess of 700 mcg. per day of total coproporphyrin and the total uroporphyrin content was quite variable, ranging from 35 to 120 mcg. per day. When this individual's urine was worked up in the usual manner with ion exchange columns the resulting pyrrole containing solution was a brilliant yellow. When this yellow solution was reacted with Ehrlich's reagent a slow reaction took place over approximately two hours. The solution was a typical red-purple

color of a pyrrole reacting with Ehrlich's reagent. When larger volumes of urine were processed and the excess 2,4-pentanedione removed and the yellow color extracted with chloroform and carried through the same scheme as that described on squirrel's urine, it was found that a series of similar findings were obtained. The yellow containing aqueous solution could be acidified with hydrochloric acid to a pH of 2 or below and slowly decolorized. After decolorization the reaction of the modified Ehrlich's reagent was instantanious and very strong. The decolorization process could be speeded up by adding acid to the aqueous solution and heating on a water bath for approximately 10 minutes. This solution also promptly reacted with the Ehrlich's reagent.

The yellow colored solution was made alkaline and extracted with ether. The ether phase contained very little of the yellow colored substance. When the ether was evaporated and the residue reacted with Ehrlich's reagent only a very slight reaction was obtained. The aqueous solution which retained the yellow compound was taken for chromatographic analysis and the solvent system of butanol, acetic acid, and water was used. After four hours at room temperature the chromatogram was dried and sprayed with the modified Ehrlich's reagent. Only one spot was seen which rapidly disappeared. This spot did not correspond to that of a pyrrole resulting from the condensation of 2,4-pentanedione and delta-aminolevulinic acid.

When large samples of this urine were processed and after the pyrrole had been formed, the resulting solution was divided into several portions. The pH of one portion was adjusted and the excess 2,4-pentanedione extracted and the resulting solution acidified to a pH of 1 with

hydrochloric acid. This was set in the refrigerator and after several days it was noted that a large number of needle-like crystals had formed. When this solution was filtered, the crystals were not retained either on filter paper or a centered glass filter and the filtrate could be re-refrigerated and the crystals would reform. Repeated efforts to isolate the crystaline material was to no avail as it appeared that these crystals might be hydrated and rapidly loose their water of hydration in the filtering process. When the supernatant solution from the crystals was carefully decanted and the crystals allowed to remain in a smaller volume of the solution, it was found that the supernatant solution was much lighter in color. This supernatant would give a positive Ehrlich's reaction, although much less intense either after heating in the presence of an acid or over a long period of time in the presence of a modified Ehrlich's reagent. Other patients whose urine showed this phenomenon of turning yellow after appropriate treatment had identical symptomatic findings but on no occasion was it possible to form crystals from the pyrrole containing solution. However, by treating the yellow pyrrole containing solution with acid and subjecting this to heat it was possible to convert the solution to one which was water clear and which subsequently would give a positive Ehrlich's reaction. Repeated chromatography of these specimens revealed only a single spot with one exception. This one exception was the urine of a young child who was suffering the second attack and who had previously showed only a urine which contained one substance by chromatographic analysis and this was the substance which corresponded to the compound which was responsible for the yellow appearance of the pyrrole containing solution. During the second episode, this individual's urine

showed a second spot which corresponded with that of the pyrrole formed from delta-aminolevulinic acid.

The presence of porphobilinogen in the urine of those individuals suffering from acute intermittent porphyria has been reported to be a rather constant finding. This compound is known to be a precursor to the formation of porphyrins. The determination of porphobilinogen was performed on the urine of each patient studied. A consistent finding in the study of the first few patients was that the urinary porphyrins were elevated that a positive Ehrlich's reaction was obtained from the Dowex 50 columns but rarely was porphobilinogen detected. An aliquot of freshly voided urine was taken for analysis before being added to the 24-hour urine collection. Because of the extremely labile nature of the porphobilinogen, the urine was placed upon a column of Dowex 2 resin within a few minutes after excretion. The column was then washed and eluted in the usual manner and reacted with modified Ehrlich's reagent. In the patients with other chemical findings of porphyria only three of these showed a positive test for porphobilinogen. After a large number of these urines were examined, the inability to demonstrate porphobilinogen in the urine became apparent. Granick reported (by personal communication) (28) that sulfhydryl containing compounds in urine would inhibit the reaction between paradimethylaminobenzaldehyde and a pyrrole. Therefore, the Ehrlich's reagent was modified so that each 25 ml. of the reagent contained .09 grams of mercuric chloride. The added mercuric chloride should then bind with any sulfhydryl containing compounds such as glutathione or cystine in the urine and thus block any inhibition of these compounds upon the reaction of the

paradimethylaminobenzaldehyde with the pyrrole. When this modification was made, it was found that a considerably higher percentage of those patients having other chemical findings and clinical symptoms of porphyria were freely excreting porphobilinogen. A number of the patients who had previously been studied provided additional urine specimens and though they were not at the present time suffering from an attack of acute intermittent porphyria their urines showed variable amounts of porphobilinogen. This finding is in keeping with that reported by Haeger (30) that individuals who have recovered from an attack of acute intermittent porphyria still excrete considerable amounts of porphobilinogen in the urine. It is believed that the modification of the Ehrlich's reagent with the addition of mercuric chloride leads to a much more reliable result. This reagent when reacted with a known pyrrole resulting from delta-aminolevulinic acid gave the same reaction as the Ehrlich's reagent without the added mercuric chloride. When urine samples that eventually produce the bright yellow color were reacted with this modified reagent the same results were obtained. Therefore, all of the patients who had been previously studied with the original Ehrlich's reagent were re-evaluated. It should be noted that a number of the patients who excreted large amounts of porphyrin in the urine and who showed a substance that was either delta-aminolevulinic acid or similar to delta-aminolevulinic acid did not excrete porphobilinogen, although all symptoms of the patients were typical of acute intermittent porphyria.

Of the entire group of individuals studied, only three had ever shown any symptoms of skin lesions. One of these was a young girl

who had developed abdominal pain, constipation and slight impairment of the right extermities. This child was shown to excrete large amounts of porphyrin in her urine which on paper chromatography proved to be uro and coproporphyrin with the isomers that of uro I and copro III. The child's mother was of fair complexion and had always blistered when exposed to sunlight for any length of time. She had never had an episode of abdominal pain, peripheral neuropathy or any of the classical symptoms of porphyria. However, on examination her urine showed that she was excreting porphyrins in similar quantities to those of the daughter. Delta-aminolevulinic acid or a substance similar to that was also demonstrated in the urine on numerous occasions. Because of the skin lesions which were presented by the mother, a sample of blood was obtained. The blood was allowed to clot and the serum was de-proteinized with the addition of sodium tungstate and sulferic acid. The precipitated protein was separated by centrifugation and the supernatant collected. The precipitated protein was extracted with methanol and sulferic acid for 24 hours and then heated to 55°C for 30 minutes. The sulfuric acid and methanol solution showed only a small amount of fluorescence. This solution was then added to the supernatant obtained from protein precipitation and the solution was once again heated to 55° for 30 minutes. The porphyrins were then extracted with chloroform and the chloroform layer was dryed over anhydrous sodium sulfate, filtered, and evaporated to dryness. The dry residue was taken up in small volume of chloroform and this was spotted on Whatman No. 1 paper and chromatographed as described in the section on methods. The results of the chromatography showed that the methyl ester of uro I and copropor-

phyrin III were extracted from the blood of this individual.

Urines from symptomatic patients which yielded elevated porphyrin values, and in which a delta-aminolevulinic acid-like substance was obtained in the eluate from a Dowex 50 column but which gave no immediate reaction for porphobilinogen were studied in more detail. The pyrrole obtained from the Dowex 50 column was chromatographed and sprayed with modified Ehrlich's reagent. The known pyrrole from delta-aminolevulinic acid was run at the same time as a marker. In some of the urines a spot corresponding to the known pyrrole was detected and in many of the samples an additional spot with a lower Rf value was detected. The spot with the lower Rf developed color after a few minutes delay and then promptly faded.

When a solution containing more than one chromatographically detectable compound was reacted with Ehrlich's reagent and the absorption measured, it was found that a broad peak resulted between 510-535 mμ. Using delta-aminolevulinic acid hydrochloride as a standard, it was found that between 5.7 and 19.8 mg. per day of the aminolevulinic acid-like substance was excreted. Normal urines were tested and none gave over 0.5 mg. per day of Ehrlich's reacting material when there was absorption in the same range. Because it was difficult, in some urines, to wash the column entirely free of urea an aliquot of the unreacted, pyrrole containing, solution was incubated at 37°C with urease and subsequently reacted with Ehrlich's reagent and the absorption again measured. In samples containing urea the absorption was lowered but the braod flat peak between 510-535 mμ changed only slightly in shape. Maximum color development was slower in the urine samples than was that obtained with

delta-aminolevulinic acid added to urine and treated in the same manner as a test sample. The Ehrlich's reaction reaches maximum intensity after about fifteen minutes with the reagent used and then decreased after another fifteen minutes. In many instances the intensity of the colored product from urine samples continued to increase over a period of several hours. The maximum absorption peak was also shifted to a higher wave length approaching that of the pyrrole from delta-aminolevulinic acid. The color change was clearly visable to the observer.

It was noted that in some of the urines a very slow reaction occurred when Ehrlich's reagent was added to the eluate from the Dowex 2 column which should have contained porphobilinogen. After several hours a colored product was obtained that was typical of the reaction between a pyrrole and p-dimethylaminobenzaldehyde. Synthetic prophobilinogen reacted promptly with the Ehrlich's reagent under the conditions used. When synthetic porphobilinogen was added to normal urine and passed through a Dowex 2 column it also reacted promptly. When the synthetic material was added to a urine, which gave a slow reaction with Ehrlich's reagent, and the absorbance measured over a one hour period it was found that the absorbance increased rapidly for about ten minutes, as did pure porphobilinogen, and then increased more slowly for an additional thirty minutes at which time the absorbance started to decrease. With pure porphobilinogen the absorbance begins to decrease after about fifteen minutes.

When a reagent, containing 0.5 grams of p-dimethylaminocinnamaldehyde in twenty-five milliliters of 6 normal hydrochloric acid, was added to the eluate of the Dowex 2 column, which reacted slowly with the

modified Ehrlich's reagent a prompt deep blue color was formed. When the reagent containing p-dimethylcinnamaldehyde was added to synthetic porphobilinogen in either aqueous solution or normal urine, a very prompt green-brown color was formed. The absorption maximum (Figure 13) of porphobilinogen reacted with p-dimethylaminocinnamaldehyde was at 448 mμ with a minimum at 510 mμ and a smaller peak at 595 mμ with a shoulder at 644 mμ. The absorption (Figure 14) of the blue compound obtained from the urine when reacted with p-dimethylaminocinnamaldehyde showed a maximum at 644 mμ with a shoulder at 605 mμ and a minimum absorbance at about 495 mμ and a smaller peak at 445 mμ. The reagent containing p-dimethylaminocinnamaldehyde will react with urea as will p-dimethylaminobenzaldehyde but gives a pink-orange color in contrast to a bright yellow color. The absorption spectrum, of 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole when reacted with the p-dimethylaminocinnamaldehyde. was almost identical to that of porphobilinogen and the chromogenic reagent. Table 7 shows findings on patients with similar clinical symptoms suggesting acute intermittent porphyria and one normal urine sample which was typical of other normal urines studied. Table 8 lists the age, sex and clinical history of the patients whose data is shown in Table 7.

The urines that gave a slow color development between the delta-aminolevulinic acid-like substance and Ehrlich's reagent also reacted slowly with the p-dimethylcinnamaldehyde reagent. Although the pyrrol containing solution had no color, it was made acidic and heated before reaction with the aldehyde reagents and gave a more rapid color development and the absorption spectrum was identical to that of the known pyrrole.

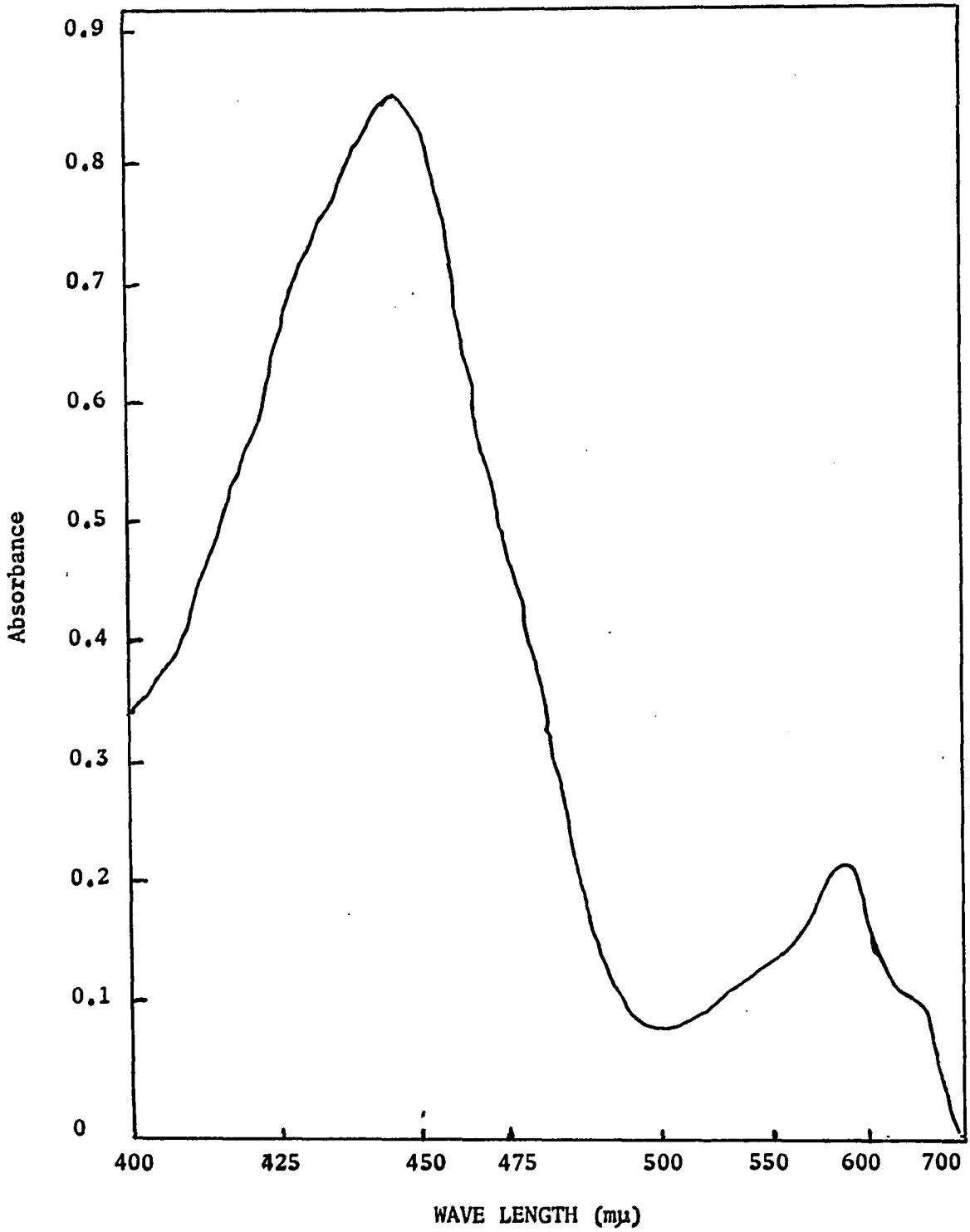


Fig. 13. Absorption spectrum of synthetic porphobilinogen reacted with p-dimethylaminocinnamaldehyde.

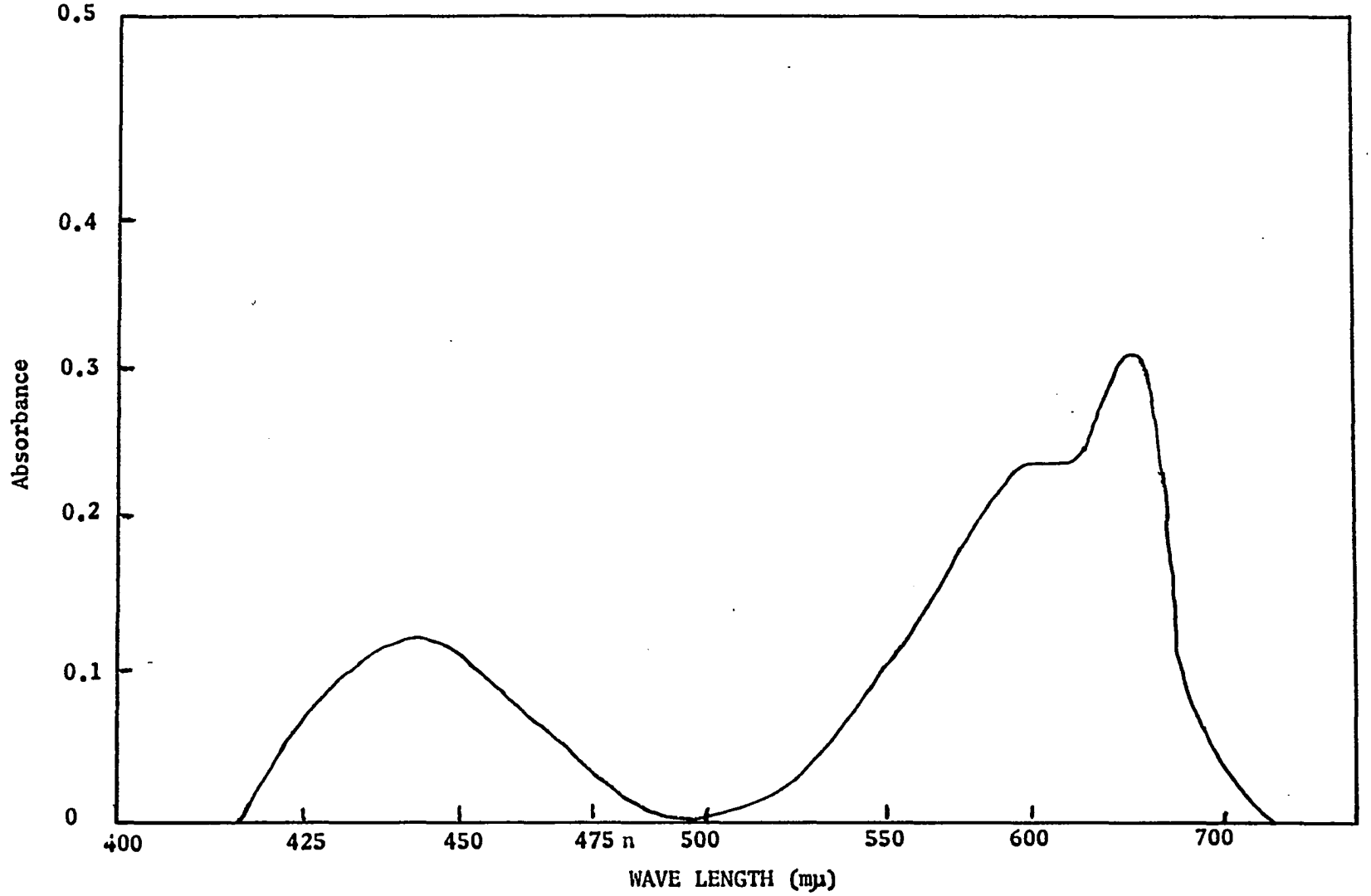


Fig. 14. Absorption spectrum of Dowex 2 eluate of human urine reacted with p-dimethylaminocinnamaldehyde.

TABLE 7

PORPHYRIN, "DELTA-AMINOLEVULINIC ACID" AND "PORPHOBILINOGEN" EXCRETION IN PATIENTS WITH SYMPTOMS OF ACUTE INTERMITTENT PORPHYRIA

Patient	Total Porphyrins/ 24 Hours	Dowex 50 Eluate with PDMBA		Dowex 2 Eluate with	
		Qual.	Quan.	PDMBA	PDMCA
C.W.H.	3948 ug	Very Pos.	13.9	Pos.	Neg.
C.W.H.	6180	Very Pos.	19.8	Pos.	Green
Li.G.	591	Very Pos.	7.8	Neg.- Slow Rx.	Deep blue
C.G.	708	Sl. Pos.	3.8	Neg.	Blue
L.G.	117	Neg.	----	Neg.	Neg.
M.G.	643	Very Pos.	8.3	Neg.- Slow Rx.	Blue
J.H.	1140	Pos.	5.7	Pos.	Green Color
B.L.	473	Pos.	6.3	Neg.-Pos. 2 hours	Blue
Do.L.	582	Pos.	11.7	Neg.	Blue
Da.L.	1433	Pos.	5.7	Pos.-Stronger 2 hours	Green
D.J.L.	211	Neg.	----	Neg.	Neg. (Reagent color deepened slightly)

TABLE 8

CLINICAL DATA ON PATIENTS SHOWN IN TABLE 7

Patients	Age	Sex	Clinical History
C.W.H.	50	Male	Abdominal Pain since 1954. No ulcer by X-ray. Right facial nerve paralysis corrected after surgery for benign hemangioma.
Li.G.	8	Female	Diabetes since age 2. Right foot drop with ascending paralysis. Treated and maintained on Thorazine since age 3.
C.G.	34	Male	Father of Li.G. No history of any medical problems.
L.G.	12	Male	Brother of Li.G. No history other than usual childhood diseases.
M.G.	32	Female	History of abdominal pain since childhood. Two previous abdominal explorations with negative findings.
J.H.	18	Male	Severe abdominal pains for four years. Many athletic injuries. Treated and maintained on Thorazine.
B.L.	22	Female	Abdominal pains since age 12. Negative abdominal exploration at age 13. Skin rash on exposure to sun. Mumps with mild pancreatitis at age 20.
Do. L.	16	Male	Well developed with no history of medical problems. Brother of B.L.
Da.L.	40	Female	Skin rash on exposure to sun. Occasional abdominal pain since childhood. Mother of B.L.
D.J.L.	34	Male	Normal control.

CHAPTER IV

DISCUSSION

The Porphyria of Fox Squirrel

In the porphyrias studied, there is a wide variation in the urinary excretion of porphyrin precursors both quantitatively and qualitatively. In the case of the squirrel, very large amounts of both uroporphyrin and coproporphyrin were excreted (9.5 mg. total porphyrin per 100 ml.) as well as delta-aminolevulinic acid and porphobilinogen. In addition very small amounts of tri-, penta-, hexa-, and heptacarboxyl porphyrins were detected chromatographically. The heptacarboxyl prophyrin has recently been referred to by Chu (8) as cutanoporphyrin because of its abundance in porphyria cutanea tarda. The finding of uroporphyrin, coproporphyrin and small amounts of the other porphyrins, coupled with the pink color of bones and teeth would seem to indicate that the porphyria of the squirrel would fall into the category of congenital porphria as manifest in man and cattle and not be classified as a physiological condition as referred to by Goldberg and Rimington (26). However, the fox squirrel shows no signs of photosensitivity as do humans and cattle. Cattle appear to be photosensitive only in areas where there is little hair (51). In this respect Kennedy (37) reported that the large amount of uroporphyrin I in Arion ater was associated with melanin and in specimens that had little melanin there was no uroporphy-

rin and suggested that the black melanin was present as a protection against the effects of light upon the animal. The fox squirrel has a very heavy black pigmentation on the tips of its hair and an abundance of hair over the whole of the body. This may effectively protect the animal from the effects of light.

One might argue that the large amounts of porphobilinogen and delta-aminolevulinic acid excreted (0.8 mg. and 1.6 mg. per day, respectively) are inconsistent with the findings in congenital porphyria. The only exception to this is the finding of Ellis et al. (17) in one herd of cattle they studied. In their study porphobilinogen was reported but not delta-aminolevulinic acid.

The chemical findings in attacks of cutaneous hepatic porphyria in man are very similar to those in the squirrel and the possible reason for the lack of skin lesions in the squirrel has already been mentioned. However, in the hepatic forms of the disease there is always impaired liver function. With the exception of those animals which developed fatty infiltration of the liver no abnormalities of the liver were grossly observable. This was also true of wild animals shot in the field and the liver immediately removed and frozen. In those few animals, which developed fatty infiltrates, no change in porphyrin excretion was noted. Also, several of the animals studied were very young and gave the same findings. One might assume that these young animals would not yet have developed liver disease.

Westall's original study on porphobilinogen indicated that it was readily converted to uroporphyrin by heat and acid solution. Porphobilinogen is converted to a random mixture of the I, II, III, and

IV isomers as shown by Mauzerall (42). Cookson and Rimington (12) have proposed a mechanism for the conversion of porphobilinogen to porphrinogens in neutral or alkaline solutions. Westall showed that the red color in urine of patients with acute intermittent porphyria was due to the conversion of porphobilinogen to a dipyrrylmethene, porphobilin, with an absorption maximum at 480 mμ. It was never shown that the porphobilinogen in the squirrel urine yielded a product with this absorption. The urine samples used in the squirrel studies were preserved with sodium carbonate in dark jars containing toluene and kept refrigerated. Even with these precautions it is possible that some of the porphobilinogen was converted to uroporphyrinogen and/or uroporphyrins although neither the II nor the IV isomer, which would also be expected, was detected chromatographically in contrast to the abundance of the I and III isomers. Furthermore, porphobilinogen was still present in urine samples when they were pooled over several weeks for studies shown in Figure 9. It would also be difficult to explain the production of the other porphyrins by some spontaneous process in the urine. This would necessitate the presence of specific decarboxylases in the urine.

The blood of the squirrel showed a normal number of reticulocytes thus suggesting normal bone marrow activity. It was felt this was not the cause of the abnormal excretion pattern.

In light of the results just described in the above material, it is felt that an abnormal porphyrin pattern has been shown to exist in the urine of the fox squirrel. The presence of uroporphyrin I and III has been shown by crystallization and melting point determinations

and by absorption spectrums (Figures 5,6). Chromatography of the free porphyrins, although not resolving the isomers, revealed uroporphyrin, coproporphyrin and three additional porphyrins. This supports the idea that artifacts were not formed during the esterification reactions to produce the esters which were used for melting point and spectrum analysis as well as isomer identification by chromatography.

That porphobilinogen was found in the urine is interesting because only in acute intermittent porphyria and severe attacks of cutaneous hepatic porphyria is this compound excreted. At these times severe clinical conditions are evident as is the case in some of the experimental porphyrias (57). Although it is difficult to assess, on several occasions the squirrels would completely lost the use of both rear legs. Several animals subsequently died and others appeared to completely recover. A similar type of paralysis has been reported by Goldberg and Rimington (26) in patients with acute intermittent porphyria. The freshly-voided urines of the squirrels gave intensely positive Watson and Schwartz tests for porphobilinogen. The more specific method of Mauzerall and Granick (43) revealed an excretion rate of 0.8 mg. per day which is extremely high considering the weight of these animals does not exceed one kilogram. Paper chromatography and spectrum analysis of the Ehrlich's reaction product also conclusively proved the presence of porphobilinogen.

Delta-aminolevulinic acid is also excreted in a limited number of human porphyrias and experimental porphyrias and in lead poisoning. This compound was also shown to be present in squirrel urine by the Mauzerall and Granick method. The level was 1.6 mg. per day, again a

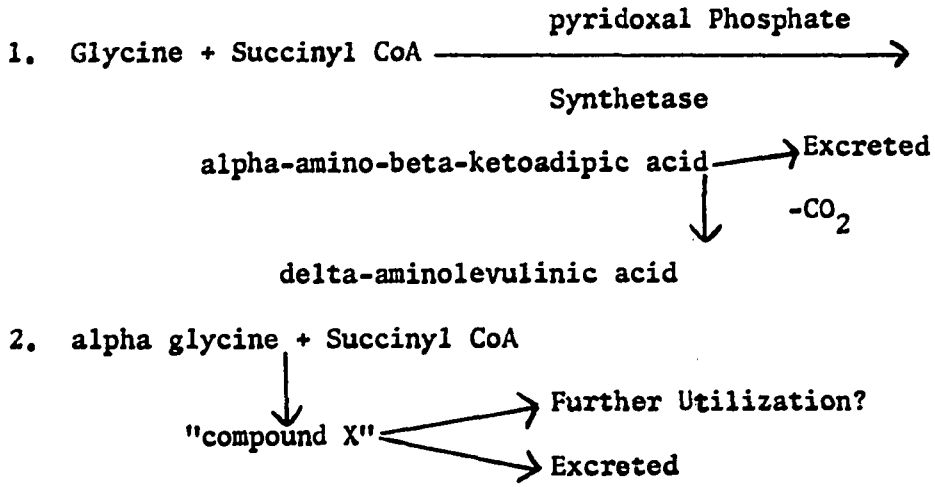
very large amount. The pyrrole formed by reacting delta-aminolevulinic acid and 2,4-pentanedione was chromatographed at the same time as the condensed eluate from the Dowex 50 column and sprayed with Ehrlich's reagent. One spot appeared with an Rf corresponding to that of the known pyrrole and also a second spot that was not identifiable. When the known pyrrole was not added to the Dowex 50 eluate but run simultaneously on another paper, it was evident that the eluate indeed contained delta-aminolevulinic acid and a second Ehrlich's positive spot. The Dowex 50 eluate was also chromatographed before any reaction to form a derivative of delta-aminolevulinic acid and sprayed with ninhydrin. Once again delta-aminolevulinic acid was present along with several other ninhydrin-positive compounds. Studies were done to rule out certain amines and amino acids as being responsible for the second Ehrlich's positive spot.

The finding of a compound, which was eluted from the Dowex 50 column, that would react with acetylacetone under the same conditions as delta-aminolevulinic acid was unexpected. It was readily extracted from a neutral aqueous solution by chloroform. Only a few milligrams of liquid was obtained from 200 ml. of urine after the solvent was evaporated. Great difficulty was encountered in solubilizing the material in water. The unknown compound would not react with modified Ehrlich's reagent promptly but did after several hours at room temperature. However, if the pH of the unknown solution was adjusted to 1 with a mineral acid and heated, the solution became colorless and promptly reacted with Ehrlich's reagent. It was not possible to elute a single fraction from the Dowex 50 column which contained the unknown material. Various concentrations of acetate were used but material could not be obtained in

a discrete fraction. It appeared in fractions both preceeding and following delta-aminolevulinic acid. The absorption spectrum of the fractions that were condensed with acetylacetone and reacted with Ehrlich's reagent gave a broad absorption maximum (Figure 11) suggesting a mixture of compounds but when these fractions were carried through the scheme shown in Figure 12 a spectrum identical to 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole was obtained.

It is interesting to consider the possible structure and origin of a compound that would lead to the above described results. Gibson et al. (23) showed that delta-aminolevulinic acid synthetase not only catalyzed the reaction between glycine and succinyl CoA but also condensed other alpha-keto CoA compounds to form amino ketones. In order of decreasing reactivity is succinyl CoA, acetyl CoA, propionyl CoA and glutaryl CoA. However, if it is the alpha position of the pyrrole derivative that is substituted and blocking the reaction with Ehrlich's reagent, this would mean that the carboxyl group of glycine was not lost when it condensed with succinyl CoA. A second possibility is that the carboxyl group is lost at the time of condensation but that the alpha carbon is substituted, as for example an amino acid other than glycine. It is possible that the succinyl CoA would react with the alpha carbon of an amino acid even though it were substituted. According to the mechanism proposed by Kikuchi et al. (38) for the activation of glycine by pyridoxal phosphate, an electron-attracting group on the alpha carbon should enhance either the loss of a proton or carbon dioxide leading to a carbanion which then reacts with succinyl CoA. However, Triebs (69) and Rimington et al. (55) have shown that it is not

necessary to have a free alpha position for the reaction between a pyrrole and p-dimethylaminobenzaldehyde. The reaction is slower but will take place. Diazotized sulfanilic acid will react with a pyrrole that has an easily replaced group in the alpha position, such as a carboxyl group. Although alpha-amino-beta-ketoadipic acid has been proposed as, and would logically appear to be, the product of glycine and succinyl CoA condensation, delta-aminolevulinic acid is the first recognizable product of the reaction. In view of the findings in this study, it is proposed that either alpha-amino-beta-ketoadipic acid is excreted in some stabilized form or an alpha substituted slycine derivative is excreted which condenses with acetylacetone yielding a yellow compound. This compound can then be converted to one which, when reacted with p-dimethylaminobenzaldehyde, gives the same product as 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole. The two possibilities may be represented in the following manner:



In studying humans with accelerated porphyrin excretion, an occasional urine was encountered which yielded a compound that gave identical results. Since none of the metabolites in porphyrin biosyn-

thesis cause symptoms associated with the porphyrias, except free porphyrins circulating in the blood causing photosensitivity, it is interesting to speculate on the deleterious effects of the proposed compound and another to be described below which was found in the human urine samples.

Abnormal Porphyrin Excretion in Humans

Although the patients studied presented with symptoms of acute intermittent porphyria, only in a very few was porphobilinogen ever demonstrated. Delta-aminolevulinic acid and a compound which behaved much like it were a consistent finding. Also elevated levels of coproporphyrin and uroporphyrin were found. The extremely low incidence of porphobilinogen was in distinct contrast to the findings of other workers Haeger (30); Watson and Schwartz, (80) and Waldenstrom (72). Because of the finding in both squirrel and human urine of a substance believed to have a substituent on the alpha carbon of the tested pyrrole, it was postulated that a compound similar to porphobilinogen but substituted in both alpha positions might be excreted. The slow reaction of the Dowex 2 eluate with Ehrlich's reagent also tended to lead to this conclusion. Prasad et al. (50) synthesized carboxyporphobilinogen and showed that heating caused the loss of the 5-carboxyl group. Rimington and Krol et al. (55) showed that this material reacted slowly with Ehrlich's reagent, before heating, at room temperature. The isomeric tricarboxylic porphobilinogen was also synthesized and called iso-porphobilinogen carboxylic acid. This compound was stable to heat.

When p-dimethylaminocinnamaldehyde was substituted for p-dimethylaminobenzaldehyde in regular Ehrlich's reagent and reacted with the

eluate from the Dowex 2 column a very rapid and intense blue color formed. This finding was repeated in a number of urine samples from patients excreting increased amounts of porphyrins and a substance that behaved similar to delta-aminolevulinic acid. The absorption spectrum of the blue compound and that of porphobilinogen reacted with p-dimethylaminocinnamaldehyde can be seen in Figures 13,14. It is believed that the compound in the urine, which appears in the eluate from the Dowex 2 column, is substituted in both alpha positions and has one or both beta positions free. The fact that it slowly reacts with p-dimethylaminobenzaldehyde suggests that it is a substituted pyrrole. The slow reaction, in comparison to the fast reaction with p-dimethylaminocinnamaldehyde, is undoubtedly due to steric hinderance. The steric effect would be less in the case of p-dimethylcinnamaldehyde because the bulky substituted benzene ring is removed further from the reactive aldehyde group.

For such a pyrrole to be present it would necessitate the presence of 2-aminopropionaldehyde or higher analog if a Knorr type reaction is to take place mediated by delta-aminolevulinic acid dehydrase. If two such molecules condensed, as do two molecules of delta-aminolevulinic acid to form porphobilinogen, the resulting pyrrole would have both alpha positions substituted and both beta positions free. If one such molecule cross condensed with delta-aminolevulinic acid or other aminoketone only one beta position would be free. The name beta-porphobilinogen is suggested for the compound excreted in porphyric urines which gives the absorption spectrum shown in Figure 14. A comparison of the reactions of the compound from human

urine and synthetic pyrroles with varying substitutions would be of great interest in clarifying its role in association with the form of porphyria studied.

CHAPTER V

SUMMARY

Studies were made on the urinary excretory products of porphyrin metabolism in fox squirrels and humans diagnosed as suffering from acute intermittent porphyria. In addition to the porphyrin isomers normally found in congenital porphyria, the known precursors of uroporphyrin i.e. delta-aminolevulinic acid and porphobilinogen were also found in abundance in squirrel urine. Such a finding has not been previously reported in the congenital type of porphyria.

An additional compound, which apparently has not been previously described as occurring in porphyric urines, was encountered and its solubility, behavior at an acid pH, its ability to form a pyrrole, and reaction with p-dimethylaminobenzaldehyde studied. It was shown that this compound could be converted to the same pyrrole as delta-aminolevulinic acid and that its Ehrlich's reaction product had the same absorption spectrum as 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole. The possible origin and nature of this compound is discussed. A compound which gave similar results was also found in some human urines in which an abnormal porphyrin excretion pattern was established.

It was found that a number of human patients with clinical symptoms of acute intermittent porphyria and abnormal porphyrin excretion did not excrete porphobilinogen. However, they did excrete a com-

pound which very slowly reacted with p-dimethylaminobenzaldehyde and very rapidly with p-dimethylaminocinnamaldehyde. The difference in the absorption spectrums of synthetic porphobilinogen and this heretofore undescribed compound is presented. It is pointed out that this compound is probably substituted in the alpha positions but has one or both beta positions free and that its slow reaction with p-dimethylaminobenzaldehyde in contrast to the rapid reaction with p-dimethylaminocinnamaldehyde, is probably due to steric effects. The necessary structure for the precursor of this compound is discussed and the name beta-porphobilinogen suggested. The form of porphyria studied appears to be different from any other previously described. The chemical findings are unique and useful in correct diagnosis of the condition and may lead to further clarification of the causes of the clinical symptoms which still remain unknown.

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