ALTERATIONS IN PURINE METABOLISM IN THE PORPHYRIC RAT

by

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A THESIS

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CHAPTER I

INTRODUCTION

In a group of human diseases known as the porphyrias, the metabolism of the porphyrins is disturbed, resulting in extreme overproduction of these compounds and their precursors. For several decades these disorders have been considered to be true diseases of metabolism,[1] and the great quantities of porphyrins formed were believed to result either from metabolic blocks or primary errors in production control. The symptoms of the diseases would then result from either a deficiency of protoporphyrin in one of its physiologically active forms, or from toxicity induced by excess circulating porphyrins. These concepts, however, were based entirely on limited observations and inferences drawn from other metabolic diseases, and lacked concrete evidence to support them. In the porphyric patient or animal, not one of the active porphyrin-containing compounds (hemoglobin, myoglobin, catalase, peroxidases, and the cytochromes) has been shown to differ structurally from the normal. Of these, only liver catalase is diminished in activity and possibly quantity.[2] Similarly, thorough investigations of the pharmacologic properties of the porphyrins and their precursors have shown these compounds to be innocuous, with the

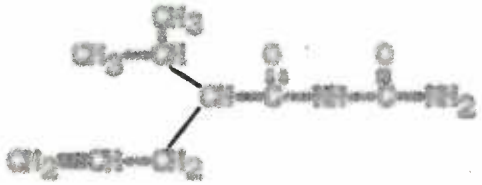
single exception that certain porphyrins and one porphyrin precursor possess definite photosensitizing properties.[3, 4, 5]

In recent years this problem has become more amenable to investigation with the discovery of drugs which are capable of producing the "hepatic" form of porphyria in experimental animals. Studies of biochemical changes occurring in this experimental disease, coupled with recent advances in our understanding of porphyrin biosynthesis, have led to the evolution in this laboratory of a new hypothesis as to the location and nature of the metabolic lesion in porphyria hepatica. Thus, it is believed that the metabolic error may lie in a non-porphyrinogenic pathway of a porphyrin precursor, rather than in the direct biosynthetic sequence leading to porphyrins. The events leading to this hypothesis and the many observations which support it will be discussed in detail presently. The work reported herein was undertaken to test this hypothesis by direct experimentation.

The Experimental Production of Porphyria

Sedormid[7] (allylisopropylacetylurea) attracted original interest as an agent which might induce porphyria as a result of Dueseberg's report[6] of a patient receiving large quantities of this drug for sedation who

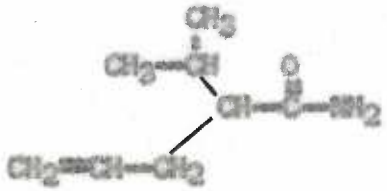
SEDORMID



developed the disease. The drug has been shown by Schmidt and Schwartz,[7, 8] Case et al.[9] and Talman et al.[10] to produce a condition in experimental animals very similar to, and probably identical with, human hepatic porphyria. Clinically, the signs and symptoms of the experimental disease in animals are similar in many respects to those noted in human subjects; i.e., ataxia, weakness, constipation, paralysis, coma, and death. Biochemically, the experimental and natural syndromes are also quite similar: 1) excessive amounts of type III porphyrins are excreted in the urine and feces; 2) porphobilinogen is found in large quantities in the urine, a finding pathognomonic for hepatic porphyria in humans;[11] 3) these pigments are found in comparable tissues at autopsy.

The use of a new drug, allylisopropylacetamide, in producing

ALLYLISOPROPYLACETAMIDE



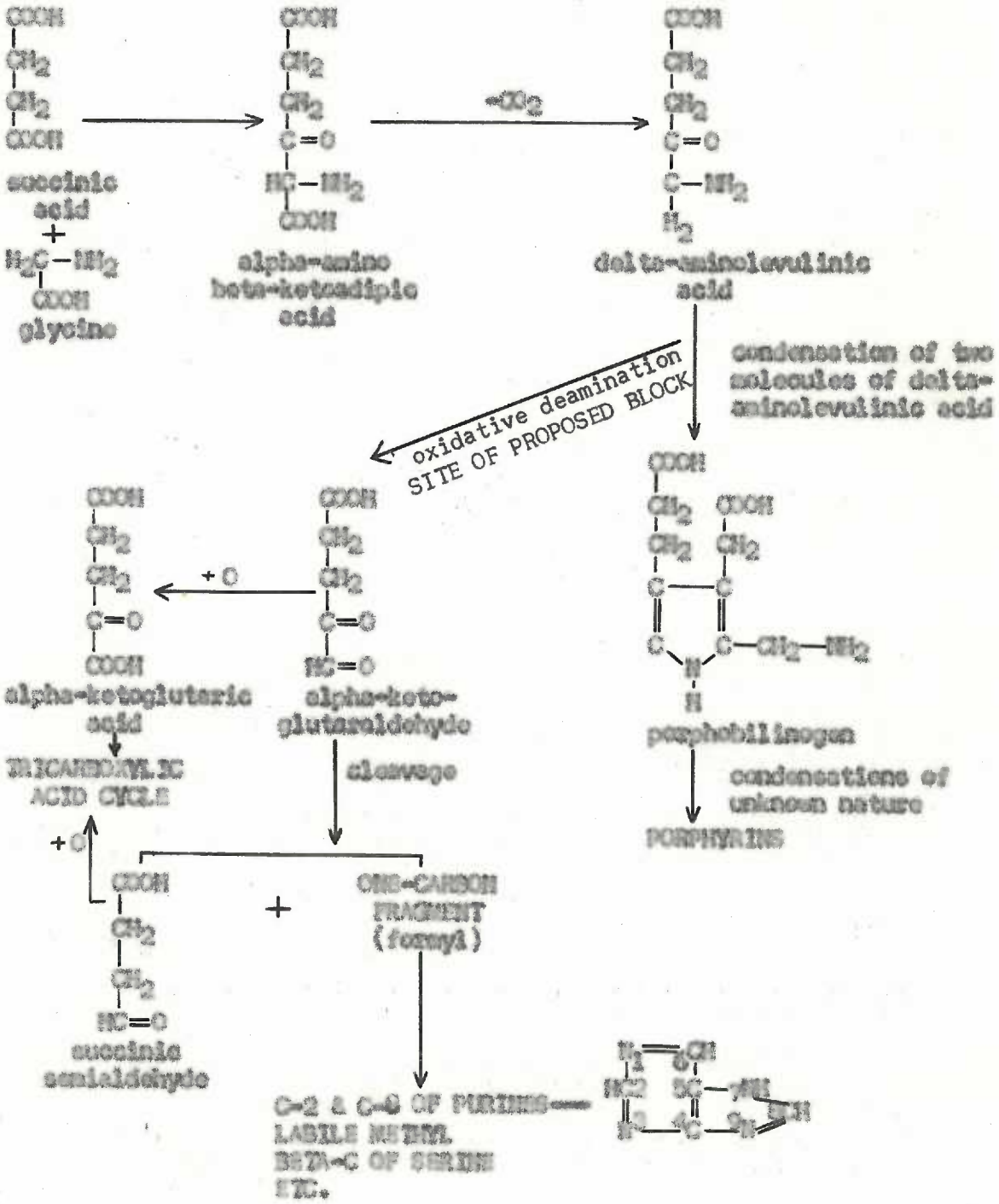
experimental porphyria has come about as a product of the search for the active functional groups of Sedormid. This new drug is more efficient in producing the disease, is a weaker hypnotic,[12, 13] and is water soluble.

Genesis of the New Hypothesis

In 1953 Shemin and Russell,[14] working at Columbia University, reported that delta-aminolevulinic acid is a common intermediate in the biosynthesis of both hemoglobin protoporphyrin and labile one-carbon fragments, some of which appear in the two and eight positions of the purine ring.[15] (See Chart I.) Coincidentally, while carrying out studies of Sedormid-induced porphyria in the chick embryo, Labbe and co-workers[16] noted a change in uric acid metabolism in the diseased embryos. These two observations--that purines and porphyrins are biochemical relatives, and that purine metabolism is altered in experimental porphyria--together with a great deal of indirect evidence, some of which has been touched upon in previous paragraphs, led the workers in this laboratory to postulate that the metabolic error in porphyria hepatica may lie in the "C_1-" producing pathway of delta-aminolevulinic acid as indicated in Chart I, and that the other pathway of this intermediary metabolite, leading to porphyrins, may function normally, except as influenced by the "C_1-"pathway block. Examination of the older observations on porphyria (presented below) make this seem the logical location for the biochemical lesion. Later experimental data have supported the hypothesis, and have singled out the purines as possibly the greatest biochemical "sufferers" from this defect in "C_1" metabolism. In support of this, it would seem very much within reason that any biochemical pathology affecting the production of compounds so physiologically vital and ubiquitous as the purines could well result in the diverse symptomatology seen in porphyria hepatica.

Chart I

DIAGRAM ILLUSTRATING THE FUNCTION OF DELTA-AMINOLEVULINIC ACID IN THE BIOGENESIS OF PORPHYRINS AND "C_1" UNITS. ALSO ILLUSTRATED IS THE PROPOSED SITE OF METABOLIC MALFUNCTION IN PORPHYRIA HEPATICA.



Indirect Evidence in Favor of the Proposed Metabolic Block

1) The old idea that there is an impediment in the biosynthesis of protoporphyrin in porphyria is difficult to reconcile with the absence of anemia as a part of the disease. Furthermore, no other quantitative or qualitative defects in protoporphyrin have been found in any of its physiologically active locations, with the possible exception noted in the case of rat liver catalase.[2] 2) The overproduction of porphyrins, porphobilinogen, and delta-aminolevulinic acid[17] in hepatic porphyria can well be explained by a block in a non-porphyrinogenic pathway of a porphyrin precursor, as proposed here. This precursor is then shunted into the porphyrin route, with a resultant over-activity of this pathway due to mass action. Also, one might conjecture a "metabolic headquarters" which would order out more of the metabolite to get the thwarted pathway moving, only to increase further the activity of the porphyrin route. 3) The possibility that some or all of the symptoms of porphyria may be due to the great quantities of porphyrins and porphyrin precursors present in the afflicted organism has been erased by the finding that, except for photosensitizing properties, the porphyrins, porphobilinogen, and delta-aminolevulinic acid are devoid of pharmacologic actions.[3, 4, 5] This experimental data directly supports several fundamental clinical observations which indicate that the above compounds have little pathogenic significance: (a) The various porphyrias, all having marked porphyrinemia in common, nevertheless present quite different clinical syndromes. (b) Patients with

porphyria hepatica may be found at times to be excreting large quantities of delta-aminolevulinic acid even when they are in clinical remission.[5] (c) An infant born to a mother mortally ill with hepatic porphyria demonstrated no ill effects, although a passive porphyrinuria during the first days of life indicated that these compounds had crossed the placenta.[18] (d) The symptoms of the disease could well be due to an abnormality of any one or all of the several biochemical systems which would be affected by a paucity of single carbon fragments. (e) As seen in Chart I, the pathway leading to one-carbon fragments could be blocked either at the deamination of delta-aminolevulinic acid or at the fragmentation of alpha-ketoglutaraldehyde. However, in order to have increased porphyrin formation the block must be at the former site, since a block at the latter location would channel alpha-ketoglutaraldehyde to the tricarboxylic acid cycle via alpha-ketoglutaric acid with no resulting backlog of intermediates to flow into the porphyrin route.

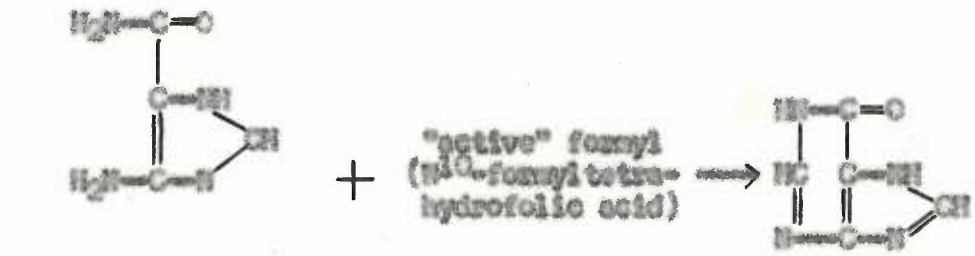
Direct Observations Supporting the Hypothesis

Several experimental observations obtained in this laboratory favor the new hypothesis by indicating a deficiency of purine synthesis in porphyria, presumably due to a lack of one-carbon fragments necessary for the *de novo* synthesis of these bases. This evidence is as follows: 1) There is a distinct decrease in the rate of appearance of uric acid in the allantoic fluid of porphyric chick embryos.[16] 2) Exogenous adenine is normally metabolized to uric acid by such embryos.[19]

3) The incorporation of 2-C^{14}-glycine into uric acid is reduced in porphyric embryos.[19] 4) Talman *et al*[20] have observed that adenine administered to porphyric chick embryos decreases porphyrin production and improves embryonic growth. 5) Sears *et al*[21] have shown that exogenous adenine decreases the mortality rate and the frequency of anatomical abnormalities and neurological symptoms among the porphyric chicks which hatch. 6) That there is a paucity of C_1-units available for purine synthesis in Sedormid-induced porphyria is supported by the observation of Labbe and co-workers[19] that the utilization of 4-amino-5-imidazolecarboxamide by the livers of porphyric rabbits is reduced. (See Chart II.)

Chart II

DIAGRAM ILLUSTRATING THE PRODUCTION OF PURINE FROM 4-AMINO-5-IMIDAZOLECARBOXAMIDE AND FORMATE[22, 23]



4-amino-5-imidazole-carboxamide

Hypoxanthine

Experimental Plan

The problem of demonstrating the proposed metabolic error in a mammalian species has centered on studies of purine metabolism in the porphyric rat. In the present work the hypothesis was tested by studying the incorporation of 2-C^{14}-glycine and of 8-C^{14}-adenine into

the liver nucleic acid purines of normal and porphyric rats. The alpha carbon of glycine becomes the delta carbon of delta-aminolevulinic acid, which is the carbon atom contributed to the C_1-pool by cleavage of this latter compound. A block in this cleavage, as proposed herein, should hinder purine synthesis. Therefore, in the porphyric animal the rate of utilization of the alpha carbon of glycine for tissue purines should be reduced, whereas the utilization of exogenous adenine, which is the only preformed purine utilized by the rat,[24] should be increased in an attempt to alleviate the purine deficiency. Also, normal or increased utilization of preformed adenine would indicate that nucleic acid synthesis per se is unimpaired.

CHAPTER II

OUTLINE OF EXPERIMENTAL METHOD

The experiment was carried out in two phases. In the first, 8-C^{14}-adenine was injected intraperitoneally into porphyric and control young rats. After twenty-four hours the animals were sacrificed and the nucleic acid purines isolated from the pooled livers. The radioactivity of these purines from the two groups of animals was compared. In the second phase, 2-C^{14}-glycine was used in a similar experimental procedure. As an indication of the presence of porphyria and as an index of its severity, liver catalase activity was determined, and qualitative porphobilinogen and quantitative coproporphyrin and uroporphyrin determinations were carried out on individual twenty-four-hour urine collections taken following injection of the radioactive material. The allantoin content of these urines was also determined, and in the case of the C^{14}-glycine-treated rats, urinary allantoin was isolated and its radioactivity determined.

CHAPTER III

MATERIALS AND METHODS

The Experimental Animal

Female Sprague-Dawley rats* weighing approximately 100 g were used. Animals of this size were chosen in order to take advantage of the more active purine synthesis occurring during growth. The use of small animals also permitted utilization of more individuals with a minimum expenditure of isotope. The animals were fed Purina Laboratory Chow and water *ad libitum* for at least three days before the beginning of an experiment. During the course of the experiments the animals were allowed free access to water, but were given no food as an efficacious alternative to pair feeding. Furthermore, it is difficult to obtain reproducible results when attempting to induce porphyria in feeding rats. Starvation was started about twelve hours before giving the first dose of allylisopropylacetamide. Four porphyric and four control rats were used in each experiment, one group following twenty-four hours after the other through the experimental procedure.

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*Obtained from Northwest Rodent Company, Pullman, Washington.

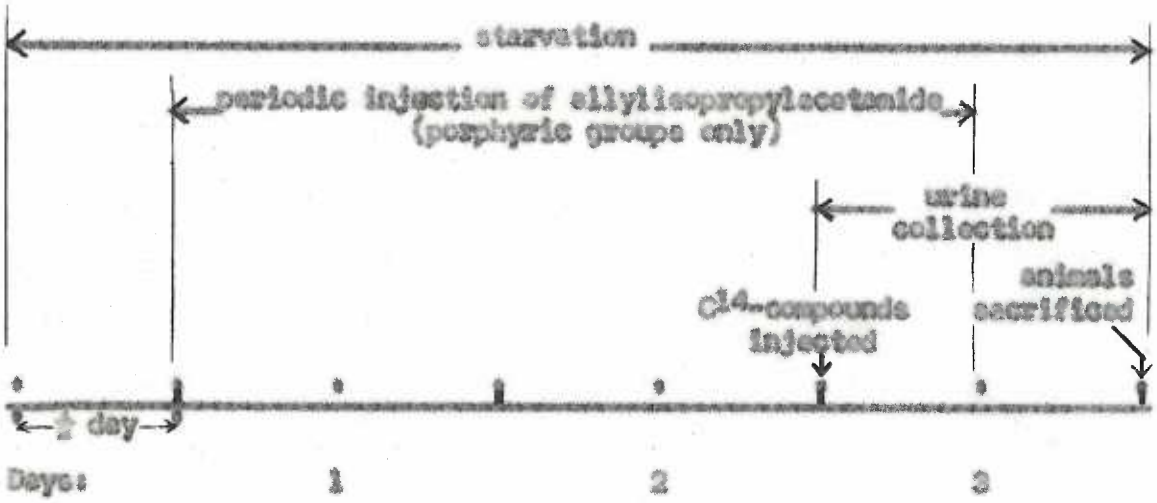
Induction of Experimental Porphyria

Porphyria was induced by subcutaneous administration of allylisopropylacetamide** as a 20 mg per ml solution in 0.45 per cent saline. The control animals were given equivalent volumes of 0.45 per cent saline. The drug was administered for three successive days; on the third day, the C^{14}-labeled compound was also injected and the rats placed in individual metabolism cages for collection of feces-free urine. Since successful induction of adequate porphyria is not always predictable, an excess number of rats was carried through the first two days of drug administration; on the morning of the third day, four rats to be carried through the remainder of the experiment were selected on the basis of clinical condition and quantity of urinary porphobilinogen. In the first phase of the experiment (C^{14}-adenine) the rats were given 40 to 50 mg of allylisopropylacetamide in two divided doses for the first two days. By the third day most of the animals were comatose, so that it was felt safe to give only one dose of eight mg to the four rats used. Even so, one of the rats expired in the closing minutes of the experimental period. In the second phase of the experiment, the drug dosage was reduced to approximately 25 mg per day in one or two doses. Chart III illustrates the management of the animals.

**Kindly provided by Hoffman-LaRoche, Inc.

Chart III

CHRONOLOGICAL MANAGEMENT OF THE EXPERIMENTAL ANIMALS



Use of Radioactive Materials

The C^{14}-tagged compounds used in this work were obtained from Nuclear Instrument and Chemical Corporation, Chicago, Illinois.

The 8-C^{14}-adenine possessed a specific activity of 1.35 mc/mM. One-tenth mc was dissolved in 16.5 ml of normal saline and the resulting solution neutralized with solid sodium bicarbonate. Two ml of this solution was injected intraperitoneally into each rat (four controls and four porphyrics); thus, the dose per rat was 12.1 uc contained in 8.96 uM or 1.21 mg of the free base.

The specific activity of the 2-C^{14}-glycine was approximately 1 mc/mM. About 0.20 mc was dissolved in normal saline to a final volume of 10 ml; each rat received 1.2 ml of this preparation intraperitoneally, the dose per rat being about 25 uc.

Collection of Urine Specimens

Immediately after injection of the radioactive material, the animals were placed in individual, all-plastic metabolism cages which are designed to separate urine and feces without the urine filtering past the fecal collection. For the most part these separations were perfect, exceptions being noted in the data tables. At the end of twenty-four hours when the animals were sacrificed, their bladders were stripped of urine by hypogastric compression, this urine being added to the collection. The urine-collecting containers and cages were washed with about 100 ml distilled water, the final volume from each cage measured, qualitative porphobilinogen tests done on each sample, and the urines stored for determination of allantoin and porphyrins. Each sample was divided for storage, a small amount of sodium carbonate being added to one portion to preserve the porphyrins, with the other portion being kept in its native acid state in order to best preserve the allantoin.

Qualitative Test for Urinary Porphobilinogen

The presence of this monopyrrole was determined by the Ehrlich aldehyde test of Watson and Schwartz.[25] The reagent, p-dimethylamino-benzaldehyde, combines with porphobilinogen to produce a red pigment which is not extracted from alkaline solution by chloroform. Results are recorded as one- to four-plus.

Quantitation of Urinary Coproporphyrin and Uroporphyrin

Coproporphyrin was quantitated by an adaptation of the "5cc method" of Schwartz et al.[26] Seventy ml of ethyl acetate was added to a separatory funnel containing a volume of urine sufficient to yield 0.5 to 2 ug of coproporphyrin, about 10 ml of water, and 5 ml of 1:1 glacial acetic acid-saturated sodium acetate buffer. After shaking, the aqueous phase, which contains the uroporphyrin, was drawn off and saved. The ethyl acetate phase, containing coproporphyrin, was washed twice with 10 ml of one per cent sodium acetate, the washes being added to the original aqueous layer. A 0.005 per cent aqueous iodine wash was used to oxidize reduced coproporphyrin chromogens. The porphyrin was then extracted with four 9 ml portions of 1.5 N hydrochloric acid, the volume adjusted to 25 ml, and the fluorescence of the acid porphyrin solution compared with a coproporphyrin standard in a Coleotron fluorimeter.

The method used for determining uroporphyrin is a modification of a procedure described by Schwartz and co-workers.[27] Its essential feature is the adsorption of the pigment onto alumina and elution with acid. The method is reasonably accurate, but it must be emphasized that any determination of the native uroporphyrin in urine gives only an approximation of the true uroporphyrin excretion picture. This is because of the instability of the pigment plus the fact that it forms spontaneously from porphobilinogen, which is also present in the urine. However, the mere presence of large quantities of uroporphyrin in urine denotes a pathological state, the demonstration of which was

the chief purpose of these determinations. It should also be pointed out that in this procedure the total fluorescence of all ether-insoluble porphyrins is measured; this has been shown to include penta- through octa-carboxyl porphyrins,[28] although the latter compound (uroporphyrin) constitutes by far the greatest portion of this group.

The procedure is as follows: About 0.5 g alumina is stirred with the combined aqueous phases remaining from the coproporphyrin determination in a 40 ml conical tip tube and centrifuge. The supernatant is discarded and the alumina washed once with 10 ml half-saturated sodium acetate and once with 25 ml distilled water. The porphyrin is then eluted with five 5 ml portions of 1.5 N hydrochloric acid and read in a fluorimeter against a coproporphyrin standard as above. The results, therefore, are in terms of coproporphyrin fluorescence units, and not actually in terms of weight units as expressed in the data. This is necessary because an accurate uroporphyrin standard is very difficult to obtain in stable form.

Quantitation of Urinary Allantoin

This was carried out by a direct adaptation from the colorimetric method of Christman *et al*[29] for blood. The procedure consists of an alkaline and an acid hydrolysis which degrades allantoin to urea and glyoxaldehyde; the latter compound is then coupled with phenylhydrazine in the presence of potassium ferricyanide to produce a red color. Results are reproducible within five per cent and recoveries give equally good results.

Isolation of Urinary Allantoin

Allantoin was isolated from the pooled urines of the C^{14}-glycine experiment for determination of radioactivity using the method of Valentine et al.[30] The procedure consists of a silver and a mercury precipitation, decolorization with charcoal, and multiple recrystallizations from hot water. Very uniform, colorless crystals were obtained. One determination of melting point was carried out, yielding an uncorrected value of 214-216°C, the reported value being 216-217°C.

In isolating allantoin from the porphyric urine, insufficient yield was obtained to allow crystallization, thus necessitating the addition of carrier.

Determination of Liver Catalase Activity

The procedure employed was developed in this laboratory from the plasma method of Dilie and Watkins.[31] It is a much less time-consuming procedure than others available. With experience, results are reproducible within ten per cent, which is entirely adequate in view of the extreme reductions in catalase activity found in the livers of porphyric animals.[32]

Reagents:

1. Concentrated phosphate buffer and saline. A 0.05 M phosphate buffer at pH of 6.8 is prepared and sodium chloride added to make an 8 per cent saline mixture.

2. Three per cent hydrogen peroxide.

3. A 0.02 N hydrogen peroxide buffer and saline solution. This is made by adding 1 ml of peroxide to 80 ml of water and 9 ml of the concentrated buffer. Prepare fresh daily.

4. A 0.01 N sodium thiosulfate solution. This is made by diluting 0.1 N stock solution.

5. A 10 per cent solution of potassium iodide.

6. A 1 per cent solution of ammonium molybdate.

7. Sulfuric acid solution, 5 per cent by volume.

8. A 1 per cent solution of starch.

Procedure:

Appropriate aqueous dilutions of liver homogenate are prepared (1:50 for porphyric livers and 1:300 for normal livers).

One ml of homogenate is added to 10 ml of the hydrogen peroxide buffer mixture in a 125 ml flask, all kept cold in an ice-water bath. After intervals of 0, 15, 30, 45, and 60 seconds, the reaction is stopped with 10 ml of sulfuric acid. (For zero time, the acid is added before the homogenate.)

Five ml of 10 per cent potassium iodide solution and 3 drops of ammonium molybdate solution are added, and the liberated iodine is immediately titrated with 0.01 N thiosulfate.

Calculations:

$$k = \frac{1}{t} \left(\log \frac{\text{blank titration in ml}}{\text{sample titration in ml}} \right)$$

k = reaction constant

t = time in minutes

The results thus obtained may be averaged, or if the procedure has been carried out carefully, the results may be extrapolated back to zero time in order to eliminate the enzyme-destruction factor. Multiplication of k by the dilution factor yields the Kat.f. of the tissue. That is, Kat.f. = $\frac{k}{\text{g of tissue}}$.

Isolation of Adenine and Guanine from Liver Nucleic Acids

Twenty-four hours after receiving the radioactive material, the rats were removed from their metabolism cages, weighed, and sacrificed by stunning. They were partially decapitated, allowed to bleed for two to four minutes, and the livers removed *in toto*, first severing the right atrium in order to gain more complete exsanguination. The livers were washed in distilled water, blotted, and weighed individually. The combined livers were then homogenized in a Waring Blendor with sufficient water to produce a 20 per cent homogenate. A sample of this homogenate was frozen immediately for determination of catalase activity. About 30 ml of this pooled liver homogenate was used for the purine isolation.

In the isolation procedure, nucleic acids were first obtained by an adaptation of the method of Schneider[33] in which they were extracted from the homogenate by hot five per cent trichloroacetic acid following preliminary extractions with cold 10 per cent trichloroacetic acid and hot organic solvents. The purines were then obtained by the method of Hitchings,[34] in which the nucleic acids were degraded by acid

hydrolysis and the purines precipitated as the cuprous salts. The individual purines were isolated by ion exchange chromatography on strongly acid resin as described by Abrams.[30]

The isolation procedure in detail is as follows:

The first of the procedure is carried out in 250 ml centrifuge cups, starting with 25 ml of homogenate in each. Fifty ml of homogenate (10 g of liver) yields more than enough purine for counting.

1. Removal of acid-soluble compounds:

After mixing 60 ml of cold 10 per cent trichloroacetic acid with the homogenate, the preparation is centrifuged, preferably in the cold, and the supernatant removed by decantation. This is repeated once. Undue delay at this stage results in a leathery residue which is difficult to handle.

2. Removal of lipids:

The tissue residue is suspended in 25 ml of distilled water, mixed with 100 ml of 95 per cent ethanol, and centrifuged; the residue is resuspended in 125 ml of ethanol and centrifuged. These steps are used to remove traces of trichloracetic acid remaining in the tissue residue from step 1. The residue is now boiled three times for three minutes each with 125 ml portions of 3:1 alcohol-ether. The addition of boiling stone facilitates this step; again, delay in continuing at this stage results in residues which are difficult to handle.

3. Removal of nucleic acids:

The tissue residue is suspended in 30 ml of water, mixed with 30 ml of cold 10 per cent trichloroacetic acid, and centrifuged in the

cold. This supernatant is discarded. The residue is suspended in 125 ml of 5 per cent trichloroacetic acid, heated 15 minutes at 90°C, cooled, and centrifuged. The residue is resuspended in 60 ml of 5 per cent trichloroacetic acid and centrifuged. The last two acid extracts are combined to form the nucleic acid extract.

4. Hydrolysis of nucleic acids:

The trichloroacetic acid solution of nucleic acids is made 0.4N with 4 N sulfuric acid and heated for two hours in a boiling water bath to hydrolyze the nucleic acids. This process also destroys the trichloroacetic acid.

5. Precipitation of the purines:

The acid solution is partially neutralized with sodium hydroxide, brought to pH 5-6 with sodium bicarbonate, and placed in a boiling water bath. While heating, 0.15 ml saturated sodium bisulfite solution per gram of tissue, and 0.1 ml 10 per cent $CuSO_4$. 5H_2O per gram of tissue is added. Heating is continued until the precipitate coagulates and turns light brown, usually within two minutes. The precipitate is centrifuged down and washed twice with water, ending with the precipitate in a 40 ml centrifuge tube.

6. Liberation of purines from precipitate:

The precipitate is suspended in 2 ml of 3 N hydrochloric acid, heated until dissolved (more acid is added if necessary), and 3 ml of hot water added. The tube is placed in a hot water bath and a rapid stream of hydrogen sulfide is passed through the mixture for three minutes. The copper sulfide thus formed is filtered off and washed

with hot water. The resulting purine solution is usually a faint yellow in color and should be 15-20 ml in volume.

7. Chromatographic separation of purines:

The cation-exchange resin, Dowex 50, 200-400 mesh, is prepared for use by washing by decantation several times with distilled water to remove the fine particles, once with 1 N sodium hydroxide, and finally with water again to remove excess alkali. The resin is transferred to a glass tube so as to form a column 15 to 17 cm by 2 cm, and is converted to the acid form by running through 2 L. of 2 N hydrochloric acid followed by 1 L. of 1.5 N hydrochloric acid, each at the rate of 2 ml per minute. The column must not be allowed to run dry.

The acidic purine solution obtained in the liberation of purines from precipitate is applied to the column, and just as the last of this flows into the surface of the resin, development is begun with 3 N hydrochloric acid, adjusting the flow rate to approximately 2 ml per minute. The effluent is collected in 25 ml fractions; all of the purines are obtained by the time 1.3 L. of the developing acid has passed through the column.

The location of the purines in the collected fractions is determined by the use of an ultraviolet spectrophotometer (Beckman model DU), obtaining the optical densities of the fractions at 260 mu. Each third or fourth tube is read, and others as needed to obtain the exact boundaries of the purine macrofractions. A typical example of results is presented in Chart IV. Generally, only those fractions having an optical density greater than one are included in the individual purine macrofractions.

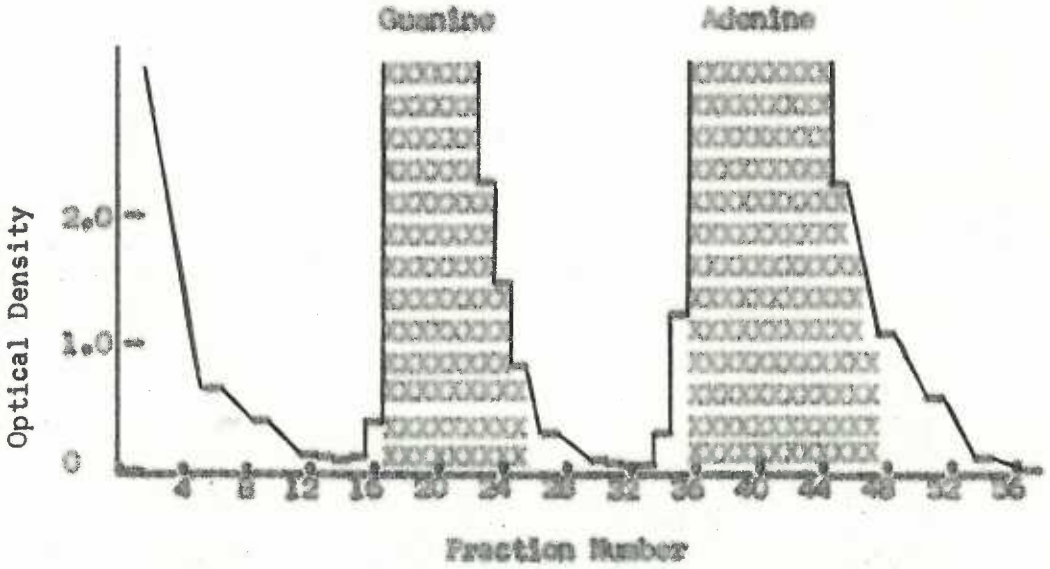
3. Concentration of the pure guanine and adenine solutions.

The acidic purine solutions are neutralized with sodium hydroxide or sodium bicarbonate and adjusted to pH 6. They are placed in a boiling water bath to which is added 0.08 ml saturated sodium bisulfite, and 0.05 ml 10 per cent $CuSO_4 \cdot 5H_2O$ per gram of tissue from which the purine was obtained. The solutions are then refrigerated until good precipitation of the copper salts is obtained; usually two hours is sufficient. Occasionally it is necessary to add a bit more alkali.

The purines are then liberated from the precipitate as in the previous step. The yield from 10 g of liver is 15 to 50 mg of each of the purines.

Chart IV

ION-EXCHANGE CHROMATOGRAPHY OF LIVER NUCLEIC ACID PURINES FROM PORPHYRIC RATS GIVEN C^{14}-ADENINE



Each fraction represents approximately 25 ml. The cross-hatched areas represent those fractions pooled to form the pure purine nucleofractions.

The presumptive adenine and guanine isolated in a trial run of this procedure were compared spectrophotometrically with pure samples. In each case the curves obtained were identical in shape and in location of maxima and minima (see Table I). These data plus the marked similarity of the chromatograms to those obtained by authors who have

Table I

ULTRAVIOLET ABSORPTION OF PURINES IN 3 N HCl

	Max.	Min.
Presumptive adenine	260 mu	228 mu
Known adenine	259	229
Presumptive guanine	250	223
Known guanine	250	223

carefully identified their products leaves no doubt as to the identity and purity of the compounds isolated by this procedure.

Quantitation of Purines in Solutions

The concentrations of the pure purine solutions was determined photometrically using an ultraviolet spectrophotometer. These solutions were diluted to approximately 5 ug per ml in 3 N HCl and their absorption compared with standards containing 5.00 ug per ml. Adenine was read at 260 mu and guanine at 250 mu. Beer's law was found to hold very well between one and 10 ug per ml.

Determination of Radioactivity

Determinations of radioactivity were made at infinite thinness using an end-window counter and 20 mm stainless steel planchets.

The purines were found to be infinitely thin when less than 0.1 mg of the hydrochloride is on a planchet. When the activity of a sample was such that an infinitely thin layer would not yield sufficient counts, heavier layers were used (up to 1.0 mg); in such cases, approximately equal quantities of the porphyric and control samples being compared were placed on planchets so as to obtain direct comparison under conditions of equal self absorption of radiation. The planchets were prepared by pipetting on appropriate volumes of the acidic purine solutions and evaporating the latter to dryness with the aid of an infra-red lamp and a slow jet of air.

Two planchets were prepared from each of the purine solutions. A total of at least 4000 counts above background were obtained from each planchet, making at least 8000 counts above background from each purine sample.

CHAPTER IV

RESULTS

General Observations on the Physical and Chemical Condition of the Experimental Animals

Tables II and III summarize the body weights, liver weights, and biochemical status with regard to porphyria of the animals used in these experiments. The quality of porphyria induced in the first phase of this work (C^{14}-adenine) was not ideal, as evidenced by the small amounts of porphyrins found in the urines (the high coproporphyrin values on the fecally contaminated urines have no significance) and small depression in liver catalase activity (less than 50 per cent). Experience has shown that porphyric rats of this size should excrete more than 25 ug of coproporphyrin per day, and liver catalase activity should be reduced below 20 per cent of normal. All this occurred even though the clinical condition of the animals was extremely poor. However, as stated previously, it was necessary to reduce the dosage of the porphyria-producing drug given to these animals on the last day, so that at the time of the above studies the rats were probably recovering from their biochemical lesion, even though their clinical condition was still extremely poor. It is clear that in the second group of animals which received smaller doses of the drug, the quality of porphyria induced was

Table II

SUMMARY OF DATA OBTAINED FROM RATS USED IN C^{14}-ADENINE EXPERIMENT

Rat numbers	Porphyrics 234	235	236	240	Ave.	Controls 241	242	243	244	Ave.
Weight at start of exp.--g. (after 12 hours starvation.)	101	103	101	97½	100½	105	110	99½	104	105
Wt. at start of 3rd day--g. (starving)	83	89½	88½	87	86½	84½	90	83½	84	85½
Wt. loss. (2 days.)--g.	18	14½	12½	10½	14	21½	20	16	20	19½
Wt. of liver--g.	5.42	5.68	6.03	4.74	5.47	2.28	2.35	2.69	2.71	2.50
Urinary porphobilinogen: 2nd day.	3+	2+	2+	2+						
3rd day. (24 hr. collection.)	2+	2+	2+	2+		0	0	0	0	
Coproporphyrin excreted on 3rd day--ug.	309	629	7.8	5.0		2.0	1.5	3.0	1.1	
Uroporphyrin excreted on 3rd day--ug.	13.6	19.2	4.7	5.0		0.7	0.4	0.6	0.3	
Catalase activity of pooled livers--Kat.i./l.					73					136

*Denotes fecal contamination of urine collection.

Table III

SUMMARY OF DATA OBTAINED FROM RATS USED IN C^{14}-GLYCINE EXPERIMENT

Rat number:	Porphyrics 257	258	260	262	Ave.	Controls 253	254	256	256	Ave.
Weight at start of exp.--g. (after 12 hours starvation.)	98	105	95½	113½	103	104½	107	96	108½	104
Wt. at end of 2nd day--g. (starving)	78	75½	75	87	79	75	76½	69½	77	74½
Wt. loss. (3 days.)--g.	20	29½	20½	26½	24	29½	30½	26½	31½	29½
Wt. of liver--g.	3.08	4.21	4.59	5.54	4.60	3.25	3.02	2.79	2.97	3.01
Urinary porphobilinogen: 2nd day.	2+	tr	2+	1+						
3rd day. (24 hr. collection.)	3+	2+	3+	3+		0	0	0	0	
Coproporphyrin excreted on 3rd day--ug.	25	32	36*	28						
Uroporphyrin excreted on 3rd day--ug.	6.6	2.9	11*	13						
Catalase activity of pooled livers-- Kat.f./g.					43.4					160

*Denotes fecal contamination of urine collection.

much better, being typical of that usually obtained. Also, these animals were strong and active.

The livers of the porphyric rats were much heavier than those of the controls, being more than twice as heavy in the first group. In order to rule out a difference in water content, the total nitrogen content of the livers of this group was estimated by the Kjeldahl method[36] using the dilute homogenates remaining from the catalase determinations. The porphyric livers were found to contain 35.0 mg N/g. and the controls contained 29.7 mg N/g. Since the starting material for these determinations was not ideal with respect to dilution and exactness of preparation, these results cannot be considered to be very accurate. However, it is certainly safe to say that the differences in mass of the livers is not due solely to water content. This was confirmed by dry weight determinations made on the second group of livers. The pooled porphyric livers of this group were found to contain 73.2 per cent water, and controls 71.3 per cent water. In view of these results, it is felt that the relatively large livers of the porphyric animals may simply reflect the reduced metabolic demands of these lethargic animals. It is well known that liver mass suffers greatly in starvation, and the liver mass of the porphyric rats, indeed, was reduced below that of normal feeding rats. Also, it will be noted, the liver mass of the second group of porphyrics, which were much more active during the experiment due to the smaller amount of drug they received, was smaller than that of the first group, and much closer to that of the controls.

Radioactivity of Liver Nucleic Acid Purines

The specific radioactivities of the liver nucleic acid purines are presented in Tables IV and V. It is seen that exogenous adenine is

Table IV

RADIOACTIVITY OF LIVER NUCLEIC ACID PURINES OF RATS GIVEN 8-C^{14}-ADENINE

	Adenine	Guanine
Controls	1.00×10^6	2.03×10^5
Porphyrics	1.72×10^6	3.70×10^5
Ratio P:C	1.72	1.82

(Counts per minute per mM.)

Table V

RADIOACTIVITY OF LIVER NUCLEIC ACID PURINES OF RATS GIVEN 2-C^{14}-GLYCINE

	Adenine	Guanine
Controls	2.14×10^4	1.57×10^4
Porphyrics	8.19×10^4	9.60×10^4
Ratio P:C	3.82	6.12

(Counts per minute per mM.)

utilized more avidly by the porphyric animal than by the normal animal. Also, the porphyric-to-control ratios of specific activity for the two purines isolated are nearly equal. As shown in Table V,

labeled glycine was also found to be incorporated into porphyric nucleic acid purines more extensively than into those of the controls.

Urinary Allantoin Studies

The 24 hour allantoin excretions of the individual animals is presented in Table VI. Included with these data is the quantity of uroporphyrin in the same urine samples. It is noted that the porphyric animals excreted appreciably less allantoin than did the controls, and also that there is a very good inverse correlation between the allantoin and uroporphyrin excretions of the porphyric animals. (See Figure V.) There was no detectable correlation of allantoin excretion rates with coproporphyrin or porphobilinogen excretions; there was no association with liver weights or weight loss of the animals; and there was no association with the clinical condition of the animals.

In the hope of throwing some light on the cause of the unexpected results of the glycine experiment, the radioactivity of the animals' urinary allantoin was determined. As has been stated, difficulties were encountered which necessitated the addition of carrier to the porphyric allantoin in the course of the isolation procedure. Due to the great amount of carrier required and the low activity of the excreted allantoin, the results are only approximate, but would indicate that the specific activity of the control and porphyric allantoins are about equal. The porphyric allantoin yielded 5.8×10^4 cpm/mM, and the control allantoin yielded 6.8×10^4 cpm/mM.

Table VI

TWENTY-FOUR HOUR URINARY ALLANTOIN (mg) AND UROPORPHYRIN (ug) EXCRETED DURING THIRD DAY OF EXPERIMENTAL PERIOD

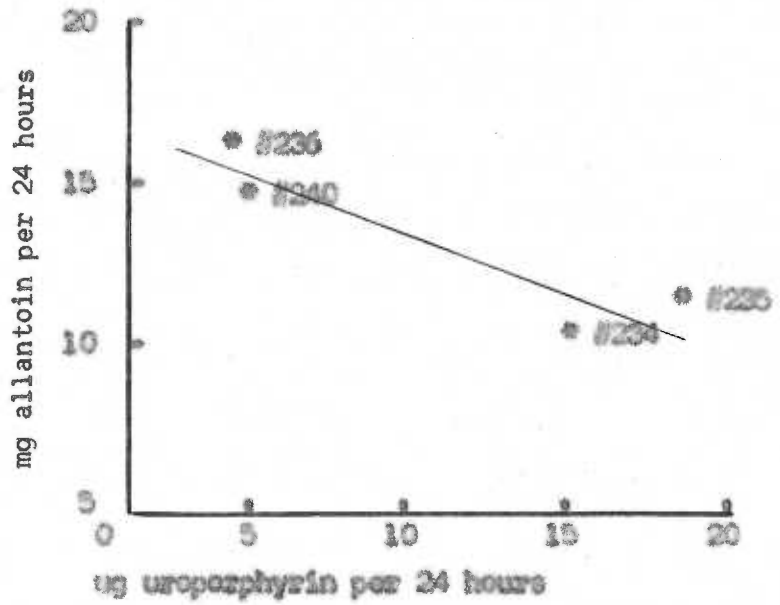
		Allantoin	Uroporphyrin
C^{14}-adenine group:*			
Porphyrics:	#234	10.2	15.6
	235	11.4	19.2
	236	16.1	4.7
	240	14.7	5.0
	Mean	13.1	
Controls:	#241	23.1	0.7
	242-244**		
C^{14}-glycine group:			
Porphyrics:	#257	16.4	6.6
	258	23.3	2.9
	260	13.5	11
	262	7.86	13
	Mean	15.3	
Controls:	#253	22.3	
	254	21.7	
	255	16.0	
	256	23.1	
	Mean	21.2	

* The allantoin determinations on this group were made after six weeks storage of the urine; therefore there may be some question of the validity of the data. It should also be noted that these animals received 1.21 mg adenine intraperitoneally at the start of the collection period.

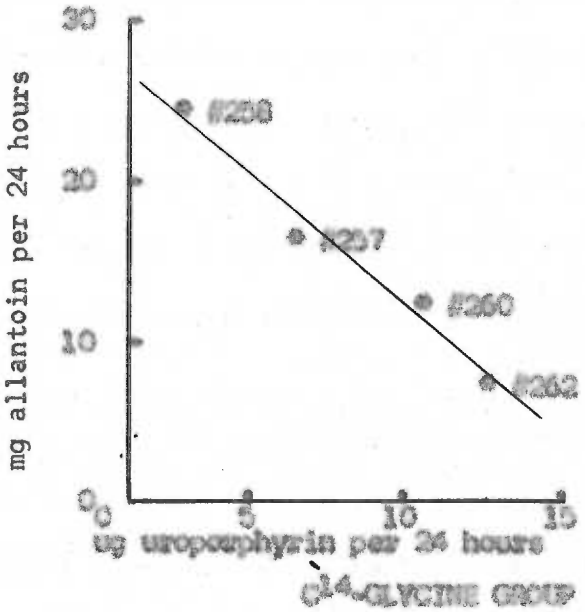
** No allantoin was found in these three samples; apparently it was destroyed by microorganisms.

Chart V

DIAGRAMS ILLUSTRATING THE INVERSE RELATION OF UROPORPHYRIN TO ALLANTOIN EXCRETION AMONG INDIVIDUALS IN THE TWO GROUPS OF PORPHYRIC RATS



C^{14}-ADENINE GROUP



C^{14}-GLYCINE GROUP

CHAPTER V

DISCUSSION AND CONCLUSIONS

Significance of Urinary Allantoin Excretion Rates --Porphyric Compared to Control Animals

The rather remarkable depression in allantoin excretion exhibited by the porphyric animals is in keeping with the proposition that purine metabolism is impaired in this variety of porphyria. However, it is also possible to explain these results on the basis of greater tissue catabolism in the controls; that is, the normal animals are much more active than the porphyrics, and therefore utilize their tissue energy stores at a much greater rate. This increased cellular catabolism could well make more purine available for degradation and excretion.

Significance of Urinary Allantoin Excretion Rates --Among Individual Porphyric Animals

As seen in Table VI the rates of excretion of allantoin by the porphyric rats varied widely among the individual animals of each group. It is seen in Chart V that this variability possessed a distinct inverse correlation with rates of uroporphyrin excretion. These data support the hypothesis by the following reasoning:

1) The animals in the experiments have demonstrated variable responses to the porphyriagenic drug; i.e., they each possessed biochemical

lesions of different severity. This is in accord with the principle of biologic variability. 2) The more severely ill animals excreted greater quantities of uroporphyrin. This is an assumption, but it would seem well within reason since uroporphyrin appears only in extremely minute quantities in the urine of normal animals, is excreted almost entirely and in relatively huge quantities via the urine in porphyric animals, and is an early product--or by-product--of the porphyrin pathways. 3) Conversely, the more severely ill animals excreted relatively less allantoin because their purine pathways were more severely impaired (in accord with the hypothesis).

It should be further noted that there was no correlation of allantoin excretion in these animals with weight loss, liver weights, or clinical condition.

As was implied in the description of methods, any one determination of urinary uroporphyrin has somewhat limited quantitative significance. However, it will be recalled that in the present work each of the determinations in a group of four was obtained under identical circumstances, thus obviating many variables. Also, in these limited observations, the excretion rates of uroporphyrin and allantoin vary so widely among animals and the observed inverse correlation is so constant that the limitations of the method can be discounted with reasonable confidence. Further substantiation of this interesting observation would be worthwhile, and should include quantitation of urinary porphobilinogen and delta-aminolevulinic acid.

The observation that allantoin excretion decreases as the severity of porphyria increases, as evidenced by urinary uroporphyrin excretion, can be taken as an additional bit of evidence favoring the proposed site of the biochemical lesion in the disease.

Interpretation of Liver Nucleic Acid Purine Studies

The results of the tissue purine studies in the C^{14}-adenine experiment came out as predicted, the porphyrics showing a greater uptake of the purine than the controls. However, the results of the C^{14}glycine experiment are quite the opposite of those which were originally anticipated on the basis of the hypothesis, making it immediately apparent that these experiments have not yielded the critical data it was hoped they would produce. Nevertheless, it is equally apparent that they provide further evidence indicating deranged purine metabolism in porphyria. There remains the task of explaining this observation on the basis of the hypothesis.

A proper thought at this point is, "What sort of results should be obtained if the theory is not valid?" If there were no biochemical lesion in the porphyric animals which directly affects their purine metabolism, one would not expect them to demonstrate such a rapid uptake of glycine and adenine into tissue purine, but rather to show uptakes approximately equal to those in the controls. On the other hand, one might contend that the high uptake of glycine in the lethargic porphyric rats was due to the lesser demand for the metabolite by energy pathways, making more available for anabolism; however, it is difficult to apply

this same reasoning in explaining the high uptake of adenine, which has no caloric value.

Since the animals were starved during the experimental period, one might also reason that, as a corollary of the greater weight loss and energy expenditure in the controls, these animals would have a lower rate of anabolism than the porphyrics. Then they would, of course, demonstrate a lesser uptake of glycine and adenine into tissue purines. This appears to be a quite logical explanation, and if true would make the observation under consideration stand as strong evidence that the proposed metabolic error of porphyria does not truly exist. However, in a recently completed experiment using chick embryos and carried out essentially in the same manner as the present work, it was observed that patterns of utilization of glycine into tissue purines are nearly congruous with those reported herein.[37] It is probably safe to assume that the same mechanism is acting in the chick embryos as in the rats to produce these results, so that even though it may be attractive to explain them on the basis of lower anabolic rate in the control rats, this explanation has little merit in either, since in chick embryos the controls are observed to grow at a slightly greater rate than do the porphyrics.[10]

With these explanations ruled out, there remains one which is compatible both with the proposed metabolic lesion and the results. Namely, that the control animals have a much higher turnover of tissue purines than do the porphyrics, and that at the time selected to sacrifice the animals the activities of the control purines had long

since reached their peaks and were well on the downgrade, whereas the activities of the porphyric purines were still near their peak values. It is a corollary of the theory being tested that such a difference in tissue purine turnover rates should exist, since, by the theory, the porphyric animals are deficient in purines as a result of a paucity of C_1 units. Also in accordance with this idea of a slow rate of purine turnover, porphyric animals do have a much depressed excretion of uric acid and/or allantoin. Furthermore, the specific activity of urinary allantoin from the porphyric rats given radioactive glycine was equal to or less than allantoin from the controls. This occurred in spite of the much depressed allantoin excretion of the porphyrics, thus indicating further that these animals were moving much less of the C^{14} into their purine pathways.

In order for this explanation involving different turnover rates to be tenable, it is necessary that the rate of replacement of tracer in liver nucleic acid purines during the first twenty-four hours after their introduction be quite rapid. Thalbert and Potter,[38] in a short-interval study of the distribution of 6-C^{14}-orotic acid given to 130-150 g rats as a single intravenous dose, found 24 per cent of the administered C^{14} to be in liver ribonucleic acids at 16 hours, and 18 per cent at 24 hours. In experiments with C^{14}-glycine using mice sacrificed 1, 2, 13, and 24 hours post-injection, Fresco and Marshak[39] have calculated apparent half-time values of 12 and 24 hours, respectively, for nuclear and cytoplasmic ribonucleic acids of liver. Unfortunately, these are the only short-term turnover studies available, and their application to

the problem at hand must be done with reservation. However, if accepted at face value, they do indicate that the turnover of liver ribonucleic acid is entirely rapid enough to lend credence to the above explanation of results. The influence of desoxyribonucleic acid upon these studies would appear to be negligible, since its turnover is relatively very slow.[40] Furst et al[41] report that the specific activity of liver ribonucleic acid of rats given C^{14}-adenine is 100 times that of liver desoxyribonucleic acid.

The possibility that a C^{14}-labeled dose of glycine would be more available to the anabolic pathways in the porphyrins because of less demand by energy pathways (a mechanism previously presented and discounted as a major factor in explaining the results) remains, and could well assist the mechanism presented above.

In conclusion, it may be stated that these studies on tissue purines have demonstrated a great aberration of purine metabolism in porphyria, as predicted on the basis of the proposed block. However, the critical data needed to prove--or disprove--existence of the metabolic block as proposed remains to be obtained. As inferred in the previous paragraph, one might well repeat the experiments carried out in the present work using shorter incubation periods. It would seem at this point, however, that the most direct attack on the problem would be to study the fate of the delta carbon atom of delta-aminolevulinic acid. A comparison of the rates of formate production from this carbon atom in porphyric and normal animals, using a formate trapping procedure such as that described by Weinhouse and Friedmann,[42] should produce quite critical data to aid

in settling the question at hand. If the rate of formate production from delta-aminolevulinic acid were found to be very low in porphyric animals, and if it were found to be normal from alpha-ketoglutaraldehyde, the compound immediately distal to the proposed block, the hypothesis as presented would be proven.

CHAPTER VI

SUMMARY

1. A hypothesis is presented which assigns the site of metabolic malfunction in porphyria hepatica to a block of oxidative deamination of delta-aminolevulinic acid, resulting in a deficiency of "C-1" fragments and their derivatives, especially purines.

2. The rates of incorporation of 2-C^{14}-glycine and of 8-C^{14}-adenine into liver nucleic acids of normal and porphyric rats are studied. Also, urinary allantoin, coproporphyrin, and uroporphyrin are determined.

3. A limited quantity of data shows a very definite inverse relation of allantoin excretion in individual porphyric rats to the severity of their disease as evidenced by urinary uroporphyrin excretion. This is in good accord with the theory presented.

4. The liver nucleic acid purines of porphyric rats given either C^{14}-adenine or glycine show higher specific activities after twenty-four hours than do the controls. This is interpreted as a further demonstration of a masked upset of purine metabolism and a delayed turnover rate of purines in porphyria; again, this is predicted on the basis of the hypothesis.

5. Critical data proving--or disproving--the hypothesis presented remains to be obtained. Experiments to produce such data are briefly outlined.

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