Experimental Porphyria. II. Type Produced by Lead, Phenylhydrazine and Light.* (19413)

S. SCHWARTZ, M. KEPRIOS, AND R. SCHMID.† (Introduced by C. J. Watson.)
From the Department of Medicine, University of Minnesota Hospital, Minneapolis

Glycine and acetate have been shown to be specific precursors of hemoglobin protoporphyrin(1), and glycine at least, is a known precursor of the uro- and coproporphyrins excreted by patients with porphyria(2). In order to study the biological interrelationships of these compounds, it would be most useful to have available an experimental animal which produced and excreted large amounts of porphyrin. Such an animal might also serve as a valuable source of radioactive porphyrins following administration of C^{14} labelled glycine or acetate. Turner has shown(3) that the fox squirrel (*Sciurus niger*), has a physiological porphyria. Preliminary studies with several of these animals indicated, however, that the practical difficulties involved in handling them limited their usefulness. Major attention was therefore directed at stimulating porphyrinuria in the rabbit, which has been shown to respond well to a variety of agents. Thus, for example, the repeated administration of phenylhydrazine to rabbits increases their urinary coproporphyrin excretion to nearly 100 μg per day from control levels of approximately 30 μg per day. Lead produces the most marked and most prolonged coproporphyrinuria of any agent known(4), especially if the rabbit is exposed to sunlight or to light from a mercury lamp(5). Peak values of up to 800 μg per day have been observed following this treatment. Finally, Pimenta de Mello has recently shown that a photodynamic dye, Rose Bengal, given in conjunction with ultraviolet light increases the urinary coproporphyrin excretion to approximately 100-300 μg per day(6). As regards previous attempts to induce porphyria in the rabbit it might be noted that sulfonal has been reported to produce an inconstant excretion of ether-insoluble porphyrin (? uroporphyrin)(7,8). No quantitative data, however, are available, and this porphyrin has not been crystallized. The isolation of uroporphyrin I from the bone marrow of rabbits treated with lead alone or with phenylhydrazine plus lead is described in a companion paper(9). Although the present studies were undertaken both to extend these tissue studies and to stimulate the urinary excretion of porphyrins to as marked a degree as possible, their most interesting result has been the production in rabbits of a temporary condition which has chemical features characteristic of human porphyria. It is the purpose of the present communication to describe the features of this experimental "porphyria" of rabbits.

Methods. The recently described ("5 ml") method(10) was employed for the determination of urinary coproporphyrin. Since rabbit urine is normally alkaline, no sodium carbonate preservative was used. Values obtained by this method averaged about twice those observed in duplicate samples which employed our previously used ("long") method. This is similar to our experience with human urine(10). Urinary uroporphyrin was determined in 5-10 ml samples of urine as follows: The urine was barely acidified with glacial acetic acid and heated in a boiling water bath for 30 minutes to convert uroporphyrin precursors. Five ml of buffered acetic acid (4 volumes glacial acetic acid plus 1 volume of saturated aqueous sodium acetate) and 30 ml of water were added and the solution shaken well with 100 ml of ethyl acetate. The ethyl acetate was washed once with 30 ml of 0.005% aqueous iodine solution and 3 to 6 times with 30 ml portions of 3% sodium acetate (or until no further red fluorescence was extracted by the wash solution). The ethyl acetate, containing the coproporphyrin,

* Aided by grants from the Atomic Energy Commission, Contract Nos. AT(11-1)-108, and AT (11-1) -100, and from the Graduate School, University of Minnesota. Presented in part before the Central Society for Clinical Research, Nov. 2, 1951.

† Clinical Fellow, American Cancer Society.

was discarded,‡ and the washes were added to the initially extracted urine for further study of the uroporphyrin. Most of the data reported here employed the Sveinsson method (11) for further purification and concentration of the uroporphyrin for fluorimetric analysis. Because variable and often considerable losses were encountered with this method another procedure has been employed more recently. The combined aqueous solution was filtered through an approximately 5 cm high and 2 cm wide column of aluminum oxide (Merck: for chromatography) to adsorb the uroporphyrin. The aluminum oxide was next washed with half-saturated sodium acetate solution until essentially no further blue-green fluorescence was removed as determined by a blank reading by fluorimetry. After further washing with about 20 ml of water, the uroporphyrin was eluted with 1.5 N HCl, and its concentration determined fluorimetrically(10). Because the uroporphyrin standards are relatively unstable, it is suggested that fluorimetric analyses be made by comparison with a standard solution of coproporphyrin which has been calibrated with freshly prepared solutions of uroporphyrin. Data on the accuracy and limitations of this procedure will be described in a separate communication. It might be noted here, however, that there is considerable error at low levels of uroporphyrin concentration as found in normal rabbit urine because of nonspecific blue-white fluorescence. At higher concentration levels (*i.e.*, above approximately 50 μg per day) a distinct red fluorescence is visible in the final hydrochloric acid eluate. The coproporphyrin isomer ratios were determined by the previously described fluorescence-quenching method(12). The presence of urinary porphobilinogen was ascertained by the chloroform insolubility of the Ehrlich-aldehyde compound(13). The further separation of the porphobilinogen into two components is described in the experimental section. The tissue copro- and protoporphyrins have been determined by the method previously described(14). The uroporphyrin present in the initial 3% sodium acetate washes was analyzed by the methods described above for the urinary uroporphyrin.

‡ Variable amounts of coproporphyrin were lost on boiling; hence a separate analysis of unboiled urine was employed for this porphyrin.

Experimental. A total of 69 rabbits have been treated with various combinations of phenylhydrazine, lead, Rose Bengal, and light. Twelve died within 24 hours after receiving lead intravenously. The following studies were made in the remaining rabbits: urinary porphyrins in 46, erythrocyte porphyrins in 27, marrow porphyrins in 23, and liver porphyrins in 10. Qualitative porphobilinogen reactions were studied in approximately 1/3 of the animals.

1. *Urinary porphyrins.* Detailed studies will be described for Rabbit No. 1 to illustrate the treatment and results obtained. These results are at least qualitatively typical of those observed in all rabbits given lead and other agents noted above. Rabbit No. 1 was injected with 20-40 mg of phenylhydrazine 8 times over a period of one month. He was then injected intravenously with 20 mg of lead acetate per kg of body weight, his back was shaved, and he was at once exposed for 30 minutes to unfiltered light from a CH-4 mercury arc lamp. Following a one-day period of anuria his 24-hour urinary coproporphyrin excretion rose to 1823 μg. Isomer analysis of this porphyrin showed it to be es-

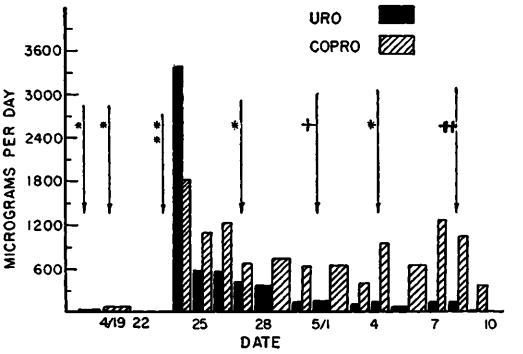

FIG. 1. Excretion of coproporphyrin and uroporphyrin in a rabbit treated with phenylhydrazine, lead and light. * 40 mg phenylhydrazine-HCl, subcutaneously (7 doses, 20-40 mg each, injected prior to 4/19). ** 40 mg phenylhydrazine-HCl, 20 mg lead acetate/kg body wt, and ½ hr light exposure (CH-4 mercury lamp). + ½ hr light exposure. ++ 20 mg lead acetate and ½ hr light exposure.

TABLE I. Peak Urinary Porphyrin Values in Different Groups of Experimentally Treated Rabbits.

Group	No. rabbits	Copro—μg/day Range	Copro—μg/day Mean	Uro—μg/day Range	Uro—μg/day Mean
1. Normals	24*	14–86	41	8–49	25
2. R.B.‡ + light	7	58–158	86	9–38	23
3. Phen.§	11	29–118	78	14–104	40
4. Phen. + light	3	64–144	96	35–64	45
5. Pb	6	301–773	465	—†	124
6. Phen. + Pb	4	227–952	532	150–503	281
7. Phen. + Pb + light	12	358–1823	726	36–3466	625
8. Phen. + Pb + R.B. + light	11	429–1041	755	50–2280	466

* Total of 39 individual analyses. Mean coproporphyrin value = 26 μg/day; mean uroporphyrin value = 17 μg/day. As noted in text, the latter values are undoubtedly higher than the true values.

† Combined analyses for uroporphyrin on only 2 rabbits.

‡ Rose Bengal, 50-100 mg/kg intrav.

§ Phenylhydrazine –HCl, 2-17 subcut. injections, 20-40 mg each.

sentially all type III isomer. However, the major portion of the urinary porphyrin, amounting to 3466 μg, was unlike coproporphyrin in being insoluble in ether or ethyl acetate. This porphyrin was isolated as the methyl ester following purification by calcium carbonate chromatography(12). The long, wavy, hairlike crystals melting at 280-282° (corrected) established its identity as uroporphyrin I, the same isomer which is present in the urine of patients with the erythropoietic (congenital or photosensitive) type of porphyria(15). This was confirmed by decarboxylation of the uroporphyrin and isolation of the resulting coproporphyrin I whose methyl ester had a melting point of 246-250° (corr.). Isomer analysis of the mother liquor indicated that it too contained only coproporphyrin I.

Daily analyses of the urinary copro- and uroporphyrin were made with the results shown in Fig. 1. It will be noted that the uroporphyrin values, after rising sharply, fell rapidly while those of coproporphyrin fell more slowly. Secondly, it is seen that the administration of more phenylhydrazine, light or lead had relatively little further effect.

Urinary porphyrin findings in the other rabbits are summarized in Table I in terms of the range of peak values and their means seen with each type of treatment. As shown in this table, markedly elevated values for both porphyrins have been observed only following the combined use of lead with the various other agents noted. Only moderate coproporphyrinuria and inconstant increases in uroporphyrin excretion were produced by phenylhydrazine, while administration of Rose Bengal plus light affected the excretion of only coproporphyrin. Because of the large spread of values within each group and because of the many variables including differences in age, sex, dose, and time of year, no attempt is made at this time to interpret too rigidly the differences between the various lead-treated groups.

It should be pointed out that chromatographic analysis of the methyl esters of both porphyrin fractions revealed the presence of very small amounts of other unidentified porphyrins. The analyses are therefore not entirely specific for copro- and uroporphyrins.

The large majority of the urinary coproporphyrin was excreted in the free form. This was established first by spectroscopic observation, and secondly by the fact that about 90% of this porphyrin was extracted readily from ethyl acetate by 0.3 N HCl. The remaining porphyrin was clearly a metal complex, with absorption maxima at 538 and 578 mμ. This was extracted by 1.5 N HCl. This behavior is characteristic of zinc-coproporphyrin. In several instances, the "native" coproporphyrin and its non-fluorescing precursor were determined separately(16) in urine freshly removed from the bladder post mortem. The two were generally present in approximately equal amounts.

On 2 occasions, rosette-like crystals characteristic of coproporphyrin III were isolated

from pooled samples of urine from several rabbits in groups 6-8 inclusive (Table I). Isomer analysis showed 89-98% of the total porphyrin to be the type III isomer.

2. *Urinary porphobilinogen.* The presence of a strongly positive Ehrlich reaction was noted in the urine of the first rabbit and confirmed in all rabbits studied subsequently. The red aldehyde compound formed was identified as porphobilinogen because of its chloroform insolubility, absorption spectrum (maxima at about 528 and 566 mμ§), and other properties as noted below. The porphobilinogen from several of the rabbits was purified by aluminum oxide chromatography as follows:¶ An equal volume of acetone was added to the combined extracted urine (unboiled) and the 3% sodium acetate washes of the ethyl acetate. This was filtered through a column of aluminum oxide (Merck: for chromatography) and the adsorbed porphobilinogen eluted with 3% acetic acid in water. When the eluate no longer gave a positive Ehrlich-aldehyde reaction, the column was washed with 35% acetic acid. A second Ehrlich-positive fraction was then obtained. The red aldehyde compound was not extracted by chloroform from either fraction. The second fraction, however, differed from the first in being completely extracted by N butyl alcohol. This difference is not related to the difference in acetic acid concentrations since addition of acetic acid to the former eluate, or dilution of the latter with water does not alter this property. A similar separation has been observed in urine from a patient with intermittent acute porphyria. The significance of this porphobilinogen fractionation is being investigated further. No attempt has been made to estimate the amount of these porphobilinogens quantitatively. It was apparent, however, that their concentration was very low in the urine of rabbits treated with lead alone. In these rabbits the presence of porphobilinogen could be established with certainty only after concentration and purification by aluminum oxide chromatography. The additional administration of the other agents described, however, resulted in definite porphobilinogen reactions which persisted for several weeks.

3. *Tissue porphyrins.* In addition to the urinary studies, we have been especially concerned with determination of porphyrin concentrations in bone marrow, circulating red cells, and liver, because of the finding that the bone marrow appears to be the chief source of porphyrin in porphyria erythropoietica (congenital porphyria) whereas the liver seems to be the main source of localization of porphyrin in the hepatic forms of this disease as seen in acute intermittent and mixed porphyria(15). The companion paper(9) has discussed the effect of lead and of phenylhydrazine plus lead on bone marrow and red cell porphyrins. The general finding of elevated values has been confirmed for these two types of treatment. Highest bone marrow values have been found in 2 rabbits given Rose Bengal and ultraviolet irradiation in addition to phenylhydrazine and lead; coproporphyrin values of 1793 and 2084 μg per 100 g were observed respectively, while the uroporphyrin concentration in one rabbit was over 1500 μg %.

There was considerable variation in the amount of porphyrin in the bone marrow and circulating erythrocytes in different rabbits

TABLE II. Erythrocyte Coproporphyrin Concentration (μg %) in Rabbits Treated with Phenylhydrazine, Lead, and Light (Autumn, 1951).*

Day	Rabbit No. 56	59†	61	63	64	65†	66†
1	5	5	7	—	—	10	—
2	11	—	13	6	10	—	9
3	24	76	26	—	—	7	—
4	21	11	20	11	13	—	4
5	—	—	—	—	—	3	—
6	14	11	6	6	4	—	—
9	4	2	3	—	—	—	—

* Avg value in 13 normal rabbits = 2.6 μg %.

† A single injection of 30-40 mg lead acetate IV on day 0 followed repeated subcut. inj. of phenylhydrazine. The other rabbits were exposed, in addition, to unfiltered light from a CH-4 mercury lamp. No more than trace amounts of ethyl acetate insoluble porphyrin was noted in any instance. Analyses were performed on red cells from 6-9 ml of whole blood.

§ Because the absorption bands are relatively broad and somewhat unstable, these measurements are to be considered approximate only.

¶ The authors are indebted to Mr. Page Edmondson for his assistance in these studies.

TABLE III. Erythrocyte Protoporphyrin Concentration (μg %) in Rabbits Treated with Phenylhydrazine, Lead, and Light (Autumn, 1951).*

Day	Rabbit No. 56	59	61	63	64	65	66
1	183	155	122	—	—	100	—
2	418	—	309	179	210	—	378
3	816	1237	631	—	—	340	—
4	912	686	590	364	355	—	444
5	—	—	—	—	—	387	—
6	537	559	366	455	327	—	—
9	533	343	321	—	—	—	—

* See Table II for protocol.
Avg value in 13 normal rabbits = 54 μg %.

treated similarly. In general, erythrocyte coproporphyrin values were much lower in the autumn of 1951 than in the spring. As noted previously(9), highest values were observed during the first 3-4 days after administration of lead, as shown in Tables II and III.

In this regard it is of interest to note that Sumegi and Putnoky(17) described increased porphyrinuria in lead-poisoned rabbits in the spring but not in the autumn, and Seggel(18) described a similar phenomenon for fluoroescytes in lead-poisoned rabbits. This possible seasonal relationship deserves further investigation.

Fluorescence microscopy study of fresh unstained smears of bone marrow revealed the presence of numerous red-fluorescing cells on exposure of the smear to the 405 mμ light from an AH-6 mercury lamp. These studies will be described in more detail elsewhere.

Two rabbits have been injected with glycine-2-C^{14} following treatment with phenylhydrazine, lead, Rose Bengal, and ultraviolet light. Results of these isotopic studies will be described elsewhere. Among the most interesting findings was the separation of 7 red-fluorescing zones on chromatography of the esterified bone marrow porphyrins. Here too, then, the analytical separation of the porphyrin into copro-, uro-, and protoporphyrins is not entirely specific.

Complete liver porphyrin analyses have been performed in but 10 instances. In no case have more than trace amounts of uroporphyrin been found. The coproporphyrin concentrations were generally normal, being elevated above 20 μg % in but one instance; a value of 268 μg % was observed in a rabbit treated with phenylhydrazine, lead, Rose Bengal, and ultraviolet light. This rabbit died during the night, 12-20 hours after treatment. All other analyses were made at least 2 days after administration of the lead.

Discussion. The excretion of uroporphyrin has generally been considered abnormal, due as Garrod termed it, to an "inborn error of metabolism"(19). Recently, however, Nicholas(20) and others have renewed Fischer's earlier claim(21) that traces of a uro-type porphyrin are present normally in human urine. We have confirmed this finding in urine from normal rabbits, rats, and dogs, but have not isolated the porphyrin in crystalline form.

As pointed out in the previous section, phenylhydrazine alone may increase the urinary excretion of uroporphyrin, while much greater increases are observed following treatment with lead and ultraviolet light. It is not unlikely, too, that methods now available will demonstrate increased amounts of uroporphyrin in a wide variety of clinical conditions. If the presence of uroporphyrin remains the decisive criterion in the diagnosis of porphyria, one might ask, "At what concentration level shall we place the dividing line in defining porphyria?" Actually, it would seem that this diagnosis is best based on the simultaneous presence of certain specific clinical *and* chemical features. Hence the tentative use of ditto marks around "porphyria" in the title of the report, though from a purely chemical point of view the characterization of this experimental condition as porphyria is not an unreasonable one. We have thus far made no clinical studies except to note that several of the rabbits have developed paresis or paralysis of the hind limbs. Whether this is related only to the plumbism rather than the "porphyria" is not clear.

It will be noted that these rabbits exhibit certain features of each of the three types of human porphyria. They resemble cases of erythropoietic ("congenital" or "photosensitive") porphyria in excreting uroporphyrin I, mainly in the free form rather than the zinc complex, and in showing high concentrations of porphyrin in the bone marrow with rela-

tively little in the liver. On the other hand, they are like cases of "intermittent acute" and "mixed" forms of hepatic porphyria in exhibiting a positive porphobilinogen reaction (though the porphobilinogen reaction is sometimes negative in mixed porphyria) and in excreting coproporphyrin which is predominantly the type III isomer. Their urine also contains increased amounts of urobilinogen for several days after administration of lead. They thus show evidence of liver dysfunction which is also present characteristically in cases of "mixed" porphyria.

Like erythropoietic or congenital porphyria patients with splenomegaly and hemolytic anemia, the rabbits have a hemolytic anemia due to phenylhydrazine, with associated stimulation of erythropoiesis and porphyrin production. The inhibited hemoglobin synthesis produced by lead, when superimposed on this stimulated porphyrin production, seems to be the important factor in causing the observed outpouring of urinary porphyrins and porphobilinogen.

Summary and conclusions. Treatment of rabbits with phenylhydrazine, lead, and light has been found to produce a temporary condition which is chemically similar to porphyria in humans. The following features are similar to those observed in human porphyria erythropoietica. a. Uroporphyrin I is excreted in the urine. Values of up to 3466 μg per day have been observed. b. The major portion of the urinary porphyrin is excreted as the free form rather than as the zinc complex. c. Bone marrow uro- and coproporphyrin values were elevated to 1500-2000 μg % as compared to normal values of approximately 0 and 5-10 μg % respectively. d. Liver porphyrin concentrations are generally normal.

The following chemical features are characteristic of porphyria hepatica. a. The urinary porphobilinogen reaction is positive. This porphobilinogen has been fractionated by aluminum oxide chromatography into 2 components. The aldehyde of only one of them is extractable by N butyl alcohol. b. The increased excretion of urinary coproporphyrin is nearly all due to the type III isomer. Values ranging up to 1823 μg per day have been observed.

The authors are indebted to Dr. C. J. Watson for his helpful support and encouragement of these studies.

1. Shemin, D., and Wittenberg, J., *J. Biol. Chem.*, 1951, v192, 315.

2. a) Grinstein, M., Aldrich, R. A., Hawkinson, V., and Watson, C. J., *J. Biol. Chem.*, 1949, v179, 983; b) Neuberger, A., Muir, H. M., and Gray, C. H., *Nature*, 1950, v165, 948; c) London, I. M., West, R., Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, 1950, v184, 365; d) Grinstein, M., Aldrich, R. A., Hawkinson, V., Lowry, P., and Watson, C. J., *Blood, J. Hematol.*, 1951, v6, 699.

3. Turner, W. J., *J. Biol. Chem.*, 1937, v118, 519.

4. Schwartz, S., and Zagaria, R., U. S. Atomic Energy Commission, Oak Ridge, Tenn., Declassified Document MDDC-504; *Toxicology of Uranium*, A. Tannenbaum, ed., National Nuclear Energy Series, Manhattan Project Technical Section, pp. 290-326.

5. De Mello, R. Pimenta, PROC. SOC. EXP. BIOL. AND MED., 1951, v76, 823.

6. ———, PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 292.

7. Neubauer, O., *Arch. Exp. Path. u. Pharmakol.*, 1900, v43, 456.

8. Waldenström, J., and Wendt, S., *Z. Physiol. Chem.*, 1939, v259, 157.

9. Schmid, R., Hanson, B., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, ——.

10. Schwartz, S., Zieve, L., and Watson, C. J., *J. Lab. and Clin. Med.*, 1951, v37, 843.

11. Sveinsson, S. L., Rimington, C., and Barnes, H. D., *Scand. J. Clin. and Lab. Invest.*, 1949, v1, 2.

12. Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J., *J. Biol. Chem.*, 1947, v168, 133.

13. Watson, C. J., and Schwartz, S., PROC. SOC. EXP. BIOL. AND MED., 1941, v47, 393.

14. Schwartz, S., and Wikoff, H. M., *J. Biol. Chem.*, 1952, v194, 563.

15. Watson, C. J., Lowry, P. T., Schmid, R., Hawkinson, V. E., and Schwartz, S., *Trans. Assn. Am. Phys.*, 1951, v64, 345.

16. Watson, C. J., De Mello, R. Pimenta, Schwartz, S., Hawkinson, V., and Bossenmaier, I., *J. Lab. and Clin. Med.*, 1951, v37, 831.

17. Sumegi, S., and Putnoky, J., *Arch. f. Gewerbe. Path.*, 1939, v9, 566.

18. Seggel, K. Ad., *Erg. Inn. Med.*, 1940, v58, 582.

19. Garrod, A. E., *Inborn errors of metabolism*, Henry Fronde, London, 1923.

20. Nicholas, R. E., and Rimington, C., *Biochem. J.*, 1951, v48, 306.

21. Fischer, H., and Zerweck, W., *Zeit. f. physiol. Chem.*, 1924, v137, 176.

Received January 14, 1952. P.S.E.B.M., 1952, v79