Conversion of Porphobilinogen to Porphyrins by Normal Rat Liver Homogenate.* (23171)

SAMUEL SCHWARTZ AND C. J. WATSON

Department of Medicine, University of Minnesota Hospitals, Minneapolis.

Westall's crystallization of porphobilinogen (PBG) from urine of porphyria patients(1) has stimulated a number of studies which indicate that this compound is the basic pyrrole unit formed in the biosynthesis of porphyrins and of heme compounds. Chemical and other studies(2,3) have shown that it is a monopyrrole containing propionic and acetic acid side chains and an amino-methyl group on one alpha carbon atom. When heated in dilute acid, PBG is readily converted to uroporphyrin(1,2). When incubated with hemolyzed chicken erythrocytes, coproporphyrin and large amounts of protoporphyrin are formed, in addition to uroporphyrin as shown by Falk *et al.*(4). Similar results have been reported by Bogorad and Granick for PBG incubated with broken cell preparations of Chlorella(5).

The present studies were undertaken to test the ability of normal rat liver homogenate to convert PBG to porphyrin. This was deemed of fundamental interest because of the previously demonstrated primary role of the liver in the genesis of those forms of human(6) and experimental(7) porphyria characterized by the excretion of large amounts of PBG. Similar studies employing livers of Sedormid-treated rats are reported in the companion paper(8).

Methods. A. Incubated samples. The PBG employed was crystallized by a slight modification(9) of Westall's procedure, from urine of patients with acute intermittent porphyria. The livers of 3 to 6 normal adult male rats were perfused with saline until the returns were nearly colorless. Three ml of cooled 0.1 molar phosphate buffer pH 7.4 and sufficient penicillin and dihydrostreptomycin to yield final concentrations of 500 units and 500 μg/ml respectively, were added/gram of pooled rat liver. This material was homogenized in Waring blender for 2 minutes. Twenty-five ml portions were pipetted into separate Erlenmeyer flasks and PBG freshly dissolved in 5 ml phosphate buffer was added to each. The "0" time porphyrin content of the control flask was determined at once, or, in some instances, after keeping at −15°C for 12-48 hours. The other flasks were incubated at 37°C for varying periods, as noted below, and then kept at −15°C until analyzed. B. Analytical procedures. The homogenate was poured with constant shaking into 12 volumes of a 4:1 mixture of ethyl acetate:glacial acetic acid. The flocculant precipitate was removed on a medium sintered glass filter, and ground three times with small additional portions of the ethyl acetate:acetic acid mixture. (Subsequent extractions of the residual precipitate with a 19:1 mixture of methanol:sulfuric acid revealed that it contained only traces of porphyrin.) One-half ml of a 1% iodine solution in alcohol was added to the combined filtrate to convert Ehrlich negative (non-PBG) "precursors" to porphyrin (10). The total porphyrin was then extracted with successive small portions of 3 N hydrochloric acid until no further red fluorescence was demonstrated in the extract in ultraviolet light. The subsequent fractionation and quantitative analysis of the copro- and proto-porphyrin was similar to that described elsewhere for the erythrocyte porphyrins(11). The uroporphyrin present in the combined aqueous fractions was adsorbed on a column of aluminum oxide and eluted with 1.5 N hydrochloric acid for fluorimetric analysis.†

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† Different lots of Al_2O_3, from the same company may differ in their suitability for this analysis. Each lot should therefore be tested with pure uroporphyrin for quantitative recovery.

As noted in the following, small amounts of several other unidentified porphyrins are included in the proto-, copro- or uro- fractions as described above. With methods now available it is not feasible to attempt complete porphyrin fractionation in the routine analyses. No satisfactory methods are available for quantitative estimation of the individual non-fluorescing porphyrin precursors which (unlike PBG) are converted to porphyrin by iodine and/or ultra-violet light(10). The following two methods were employed for estimating the total amount of these compounds in aliquots of the primary ethyl acetate:acetic acid extract: (1) Determination of difference in fluorescence intensity of this extract before and after addition of iodine and exposure to ultra-violet light, and (2) determination of fluorescence intensity of 3 N hydrochloric acid extracts of 2 portions of the ethyl acetate, only one of which had been treated with iodine. These procedures were carried out in a dimly-lit room. For more precise analysis of the porphyrins, several of the fractions were esterified with methanol: sulfuric acid (19:1) and further purified by chromatography on calcium carbonate(12). The ratio of coproporphyrins I and III was studied by a slight modification of the previously described fluorescence quenching technic(12) and by the paper chromatographic method of Chu *et al.*(13). Copro-, and protoporphyrins were crystallized as methyl esters from methyl alcohol. Melting points were taken with a Fisher-Johns micro-melting point apparatus. The uroporphyrin fraction was further analyzed by the paper chromatographic method of Falk and Benson(14). A method later devised for quantitative estimation of PBG in the liver(8) was not available at the time the present studies were carried out. Instead, qualitative Ehrlich reactions were observed on 10% acetic acid or 1% ammonium hydroxide extracts of the homogenates. The following method was employed to determine the relative activity of cell-free versus cell-containing fractions of the liver homogenate. From a total of 360 ml, four 25 ml portions were incubated for varying periods after addition to each of 1.0 mg of PBG in 1 ml phosphate buffer pH 7.4. The remaining 260 ml was centrifuged at 3000 rpm for 20 minutes. 160 ml of opalescent cell-free supernatant solutions were removed, and the same volume of phosphate buffer containing penicillin and dihydrostreptomycin was added to the centrifugate. The latter was then mixed by homogenizing for 30 seconds. 25 ml portions of supernate and reconstituted centrifugate were incubated with PBG in the same way as described for the whole homogenate.

Results. The conversion of PBG to porphyrins by normal rat liver homogenate has been studied in 4 separate experiments. In each, considerable amounts of copro- and protoporphyrin and smaller amounts of uroporphyrin appeared. PBG disappeared progressively from the samples, being present in trace amounts at 9 hours and absent at 22 hours. The data from 2 of these studies are summarized in Table I. In a third study analyses were made only at 0, 3 and 6 hours. The 6 hour sample contained 0.4 μg of uroporphyrin, 5.7 μg of coproporphyrin, and 11.6 μg of protoporphyrin per gram of liver following addition of 200 μg of PBG/gram of liver in the homogenate.

Activity of whole liver homogenate as compared with that of the supernatant solution and centrifugate may be seen in Table II. It is evident that the amounts of porphyrin were similar with whole homogenate and supernatant solution. Relatively little conversion to copro- and protoporphyrin occurred in the centrifugate. As in the previous studies, the 9 hour uroporphyrin values exceeded the 22 hour value.

Evidence was regularly obtained for the presence of non-PBG precursors converted to porphyrin by iodine and ultra-violet light. Measurements of fluorescence intensity of the primary ethyl acetate:acetic acid extracts of the homogenates and supernates incubated for 3 to 9 hours showed 3-fold increases or more after the addition of I_2 and exposure to ultra-violet light, the gross fluorescence changing from greenish-yellow to orange or bright red. No increase was observed in the extracts from the similarly treated centrifugates. It is thus evident that over half of the total porphyrin was present in precursor form in these

TABLE I. Conversion of Porphobilinogen to Porphyrin by Normal Rat Liver Homogenate.

Incubation time (hr)	Total porphyrin recovered (μg/g liver)					
	Exp. 1			Exp. 2		
	"Uro"*	"Copro"*	"Proto"*	"Uro"*	"Copro"*	"Proto"*
0	.02	.14	.17	.2	3.3	.8
.5	.02	.4	1.9			
1.2	.08	1.25	2.7			
2	.25	5.0	5.0			
3				1.25	10.0	6.5
4	1.1	11	7.5			
9	3.5	23	19	2.9	25	16
22	1.3	36	34	.8	38	58

0.20 mg porphobilinogen added/g of liver in each series. Control samples incubated similarly without added porphobilinogen all contained less than 0.02 μg of uroporphyrin and 0.06-0.15 μg of coproporphyrin/g of liver. Protoporphyrin values in control samples rose progressively with incubation from 0.06 to 1.2 and from 0.3 to 0.8 μg/g of liver in series 1 and 2 respectively. Total recovery as porphyrin at 22 hr = 51 and 67% respectively. (Values corrected for individual molecular weights.)

* As discussed under *Methods*, it is recognized that this fractionation does not exclude small amounts of other unidentified porphyrins. This also applies to data in Tables II and III.

homogenates and supernates. (In other studies, extraction of the homogenate in the dark has shown over 90% of the porphyrin to be present in the non-fluorescent state.)

In the 2 instances (Tables I and II) in which the significant amounts of coproporphyrin were found in the "0" time samples, most of the porphyrin was present in precursor form. This suggests that significant conversion of PBG had occurred before the sample was deep frozen or during warming to room temperatures.

In addition to control data for liver alone, shown in Tables I and II, the incubation of 0.74 mg of PBG with phosphate buffer and antibiotics alone, yielded 3 μg of uroporphyrin at 9 hours and 72 μg at 22 hours. The latter value is much larger than any of the values noted for incubated homogenate. No copro- or protoporphyrin was demonstrated in this experiment at any period. Only faint traces of porphyrin (less than 0.1 μg total) were found even at 22 hours in the control samples of diluted rat blood without PBG

TABLE II. Conversion of Porphobilinogen to Porphyrinogen and Porphyrin by Rat Liver Homogenate, Supernatant "Solution," and Centrifugate.

Sample	Incubation (hr)	Fluor. increase* (%)	"Uro" (μg)	"Copro" (μg)	"Proto" (μg)
Homogenate	0	105	.2	10	3
	4	127	.5	63	15
	8	216	19	167	46
	22	45	12	270	105
Supernate	0	0	.4	4	2
	4	79	2	87	28
	8	86	10	159	67
	22	0	4	99	130
Centrifugate	0	0	.1	.3	.2
	4	0	1	13	12
	8	0	8	27	29
	22	0	43	42	41

0.2 mg crystalline porphobilinogen added/g of liver. Control uro- and coproporhpyrin values/g of liver all less than 0.02 μg; control 22 hr protoporphyrin in homogenate = 4 μg and 1.5 to 1.7 μg in other 22 hr samples. Total recovery as porphyrin in 22 hr homogenate = 52%.

* Measurement of porphyrinogen content by comparison of red fluorescence in primary ethyl acetate:acetic acid extract before and after addition of I_2 and brief exposure to ultraviolet light.

These data were presented in somewhat different form at the Ciba Conference, Ref. 15 (p. 211).

while in similar samples containing PBG only uroporphyrin increased significantly (8 μg per mg of added PBG, in 22 hours).

Porphyrins extracted from individual homogenates incubated with PBG were pooled for further analysis in 4 separate batches, depending upon duration of the incubation period. They were esterified and then purified by calcium carbonate chromatography. Chromatography of the "uroporphyrin" fraction yielded one major zone, with only a trace of a second. The "coproporphyrin" fraction yielded from 6 to 8 zones in the 9 to 22 hour samples. Coproporphyrin comprised an average of 74% (range 63 to 85%) of the total of these samples. Six zones were obtained from each of the "protoporphyrin" fractions. A quantitative fractionation in 2 instances revealed that the protoporphyrin comprised but 68 to 69% of the total porphyrin. Paper chromatographic analyses of the proto-, copro-, and uro- fractions revealed the presence of a total of at least 11 different porphyrins, the major components behaving as copro- and protoporphyrin. The behavior of the other fractions indicated the presence of uroporphyrin and of porphyrins with 3, 5, 6 and 7 carboxyl groups.

Isomer studies of the total coproporphyrins pooled from the various incubations revealed 75% to be type III at 9 hours and 58% at 22 hours by the fluorescence quenching technic. The major portion in both behaved as coproporphyrin III by the Chu method.

For crystallization, the combined coproporphyrin ester was evaporated to dryness and the residue was washed twice with 3 ml portions of ether cooled to −15°. The ether soluble porphyrin was crystallized from methyl alcohol, yielding rosettes typical of coproporphyrin III methyl ester, with a melting point of 144-145°. Small curved needles similar to coproporphyrin I were obtained on one occasion from the ether-insoluble residue, but the amount was too small for recrystallization and melting point determination. The combined protoporphyrin methyl ester was crystallized, m.p. 222°C, (proto-9-methyl ester). Analysis of the uroporphyrin by paper chromatography revealed a predominance of type III isomer in samples incubated for 9 hours, whereas approximately equal amounts of types I and III were present in the 22 hour samples.

TABLE III. Conversion of Porphobilinogen to Porphyrins in Chicken Red Blood Hemolysate.

Incubation time (hr)	Total porphyrin recovered (μg) Uro-porphyrin	Copro-porphyrin	Proto-porphyrin
.0	.1	.1	.3
7.3	82	40	30
21.0	94	81	45

25 ml of heparinized chicken blood were lysed with 220 ml H_2O, and 20 ml of 0.1 M phosphate buffer and antibiotics (as for liver homogenates) were added. 500 μg porphobilinogen were added to each flask containing 20 ml of lysed blood. Total recovery as porphyrin = 53% in 22 hr sample; recovery as copro- plus protoporphyrin = 35%.

In a final experiment a study was made of the conversion of PBG to porphyrins in chicken blood hemolysate. The results are summarized in Table III. It will be noted that while the total porphyrin recovery compared well with that obtained with liver homogenates, a much higher proportion was recovered as uroporphyrin when chicken hemolysate was used. Again, significant amounts of porphyrin precursors were noted in the incubated samples.

Discussion. The present study shows that PBG is readily converted to uro-, copro-, proto-, and other unidentified porphyrins by normal rat liver homogenate. Some implications of this finding will be discussed in greater detail in the companion paper(8) and elsewhere. Here we will consider only the relationship of these studies to the problems of heme synthesis.

Various investigators have postulated the biological occurrence of progressive decarboxylation of the 8-carboxyl uroporphyrin via the 4-carboxyl coproporphyrin to the 2-carboxyl protoporphyrin. Considerable evidence against this concept has been presented by one of us(15). The demonstration in the present studies of large amounts of precursors converted to porphyrin by iodine suggested that these presumably reduced porphyrins may be the biologically active intermediates in heme synthesis. Since completion of these studies, reports by Neve and co-workers (16, 17), Granick(18) and Bashour(19) and col-

laborators have shown that this is indeed the case.

The differing solubility properties of uro- and coproporphyrin and their respective porphyrinogens has permitted their separation and direct identification. To date this has not been possible in the case of protoporphyrin. Evidence for the occurrence of reduced protoporphyrin as a metabolic intermediate is therefore indirect only, and is derived from the fact that in some studies the amount of protoporphyrin isolated after addition of iodine exceeded the total amount of porphyrin present prior to treatment with iodine.

Studies have been made of infra-red spectra and other properties of the various porphyrins isolated from the incubated samples. These are being compared with those of porphyrins obtained by the partial decarboxylation of uroporphyrins I and III and will be reported separately.

Summary. 1. Crystalline porphobilinogen (PBG) incubated with rat liver homogenate has been shown to be converted to at least 11 different porphyrins, the majority of which is copro- and protoporphyrin, with a smaller proportion of uroporphyrin. The total amount at 22 hours incubation in each of 3 experiments was slightly in excess of 50% of the PBG added. 2. At least half of the porphyrin in the 3 and 9-hour incubated samples were present in a non-fluorescing form. These porphyrin precursors were readily converted to porphyrin by addition of iodine or exposure to ultra-violet light. 3. The major part of the porphyrins isolated was isomer type III. The proportion of type I isomer increased with continued incubation. 4. The supernatant suspension obtained by centrifugation at 3000 rpm for 20 minutes was essentially as active as the whole homogenate, in the conversion of PBG to porphyrin.

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