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Enterohepatic Circulation and Conversion of Protoporphyrin to Bile Pigment in Man* (32829)

GEORGE W. IBRAHIM AND C. J. WATSON

University of Minnesota Unit for Medical Research, Northwestern Hospital, Minneapolis, Minnesota 55407

The literature prior to 1950 on the intestinal formation and absorption of porphyrins was reviewed in relation to an earlier detailed study of this problem (1). The collected evidence for and against absorption of copro- or protoporphyrin was inadequate and indecisive. The study referred to failed to reveal any increase of urinary coproporphyrin after feeding of either of the coproporphyrin isomers, or of hemoglobin, or meat. None of these earlier studies excluded absorption and re-excretion in the bile or possible conversion to other derivatives. This would relate especially to protoporphyrin which is not excreted in the urine. Appropriate studies in subjects with bile fistulae were not available. More recently, Aziz (2) fed protoporphyrin through an inlying duodenal tube in an individual having a complete external bile fistula following operation for carcinoma of the pancreas. The administered protoporphyrin was unaccounted for on the basis of serial determinations of protoporphyrin in bile samples collected after the feeding. In the meanwhile London and co-workers (3) had shown that protoporphyrin ^{15}N given intravenously in a normal dog was converted to stercobilin but whether *via* preliminary heme formation in the liver was not determined. Quite recently (4) we have reported that protoporphyrin ^{14}C is promptly converted to hepatic heme, thence to bilirubin, after intravenous administration in bile fistula dogs. A part of the injected protoporphyrin was excreted in the bile as such, together with labeled bilirubin.

Recently clinical improvement has been reported following cholestyramine therapy in patients with porphyria cutanea tarda (5) and with erythropoietic protoporphyria (6). This stimulated our interest to seek the existence of an enterohepatic circulation (EHC) of protoporphyrin. It was believed that the problem could only be solved by the use of suitably labeled protoporphyrin (Proto-). The above-mentioned conversion of Proto-^{14}C to bilirubin in dogs (4) provided the basis for the present study. If Proto-^{14}C administered intraduodenally in man is absorbed, some fraction of the absorbed porphyrin should be converted to bile pigment in the liver and would then be detected as stercobilin ^{14}C in the feces.

Material and Methods. The study was carried out on one of us (G.I.), a normal male subject, age 37, in excellent health. Proto-^{14}C was prepared as described previously (4) from a duck red cell hemolysate system incubated with 0.1 mC of δ-aminolevulinic acid-4-^{14}C (ALA-^{14}C), see Table I. The hemoglobin Proto- dimethyl ester was isolated from duck hemoglobin by Grinstein's method (10). The free erythrocyte porphyrins in the preliminary acetone wash of the hemoglobin powder (10) were fractionated as described in a previous publication (4). The crystalline free Proto- fraction (125 μg, 178.3 × 10^6 DPM/mg) was added to enrich the benzene solution of the crystalline (Hb) Proto- (5.22 mg, 1.75 × 10^6 DPM/mg). The mixture was chromatographed

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TABLE I. Samples of Proto-^{14}C from Duck Red Cell Hemolysate[a] after Incubation with ALA-^{14}C.[b]

Sample	mg	DPM × 10^6/mg	Total DPM × 10^6
1. Total Hb Proto-, cryst.	25.7	1.75	44.9
2. Free eryth. Proto-	0.125	178.5	22.3
3. Proto- administered thru duodenal tube[c]	3.87	3.66	14.2

[a] Eight ml red cells containing 2.05 gm of hemoglobin or 68 mg theoretical yield of Proto-.

[b] Volk Radiochemical Co., Burbank, California, 1.1 mC in 1.08 mg, 15.5 mC/mmole.

[c] 5.22 mg of sample 1 + all of sample 2, mixed, chromatographed and recryst. (see "Material and Methods").

on a $CaCO_3$ column. The Proto- ester was eluted with benzene–$CHCl_3$ (10:1) and crystallized from $CHCl_3$:CH_3OH. The ester was hydrolyzed on standing overnight in 7.5 *N* HCl. The free Proto-^{14}C obtained (3.873 mg, 3.66 × 10^6 DPM/mg, total 14.2 × 10^6 DPM) was dissolved in 0.5% ammonium hydroxide and administered to G.I. through a duodenal tube. Stools were collected daily for 12 days and were frozen immediately and kept at —15°C until the time of laboratory tests. Urine was similarly collected for the first 3 days. At later dates, stool and urine samples were collected and analyzed for total radioactivity, or otherwise studied, as described below.

Stool analysis. Individual stool samples were thawed, weighed, and well mixed. A portion (approximately 5 gm) was weighed in a dish and used for urobilinogen determination (7). An equal portion was kept frozen for determination of porphyrins (8). A third portion (approximately 0.3–1.0 gm weighed on a microbalance) was used for total radioactivity analysis. The remainder of the stool sample was used for isolation of stercobilin (9) and porphyrin, as described below.

Isolation of stercobilin. The sample of feces was extracted repeatedly with 95% alcohol in a medium porosity sintered glass funnel. The alcohol extract was filtered with suction, the fecal residue being kept for isolation of porphyrins (see below). The alcohol extract was applied to an aluminum oxide column. Stercobilin and stercobilinogen remain on the column and are eluted with distilled water. After elution, the column was washed with 3*N* HCl which removes a variable, minor portion of the total porphyrin in the fecal sample. This was added to the main porphyrin fraction obtained, as described below. The water eluate was acidified with 5 ml of glacial acetic acid and stercobilinogen was extracted by repeated shaking with 10-ml portions of petroleum ether. The latter was pooled, washed with water, and shaken repeatedly with 10 ml portions of 0.002% iodine solution, thus converting stercobilinogen to stercobilin. The stercobilin enters the aqueous phase, the iodine remaining in the petroleum ether. The pooled aqueous was shaken with 100 ml of ether to remove mesobiliviolin, then acidified with sufficient 25% (w/v) HCl to make approximately 1.5 *N* after which the stercobilin hydrochloride was extracted with small portions of chloroform. The latter was filtered through a chloroform moistened paper and evaporated to a small volume in a LyoVac unit. The stercobilin hydrochloride was crystallized from chloroform acetone and recrystallized from chloroform ethyl acetate. An additional amount was obtained by the same procedure from the water eluate after the above ether extraction.

Isolation of porphyrin. The fecal residue after extraction of stercobilin and stercobilinogen with 95% alcohol was extracted with 50-ml portions of 4:1 ether:glacial acetic acid mixture. This was filtered through a medium porosity sintered glass funnel under suction. Extraction was continued until the filtrate showed no red fluorescence under UV light. The pooled filtrate was washed 3 times with 3% sodium acetate and the porphyrins were extracted by 3.0 *N* HCl. To this fraction was added the above-mentioned HCl wash of the aluminum oxide column used in the isolation of stercobilin. The pooled HCl

was neutralized with saturated sodium acetate to Congo negative reaction. The porphyrins were recycled into ether, then 1.5 *N* HCl. The latter was diluted to 10 vol. with distilled water and the Proto- or other 2-COOH porphyrins were extracted from the 0.15 *N* HCl with chloroform. The chloroform was extracted with 10-ml portions of 10% sodium hydroxide which resulted in precipitation of the porphyrin as the sodium salt at the interphase. The precipitate was separated by filtration, and when dry, dissolved off the filter paper with 20:1 methyl alcohol:concentrated sulfuric acid. This was kept in the dark overnight to permit esterification. The dimethyl esters of the porphyrins were then extracted in chloroform and the latter washed in the usual manner (10). The chloroform was then filtered through $CHCl_3$ moistened paper and evaporated to dryness in a Lyo-Vac unit. The residue was dissolved in benzene to which 10 vol. of petroleum ether was then added. The solution was chromatographed on a Celite column. The porphyrin ester was eluted with benzene and crystallized well from benzene–methanol. As indicated in the following, this was a mixture of Proto and other 2-COOH porphyrins. Paper chromatography for the carboxyl number of the porphyrins was done according to the method of Chu (11). Thin-layer chromatography of the porphyrin esters was carried out as described by Chu and Chu (12).

Radioactivity analysis, feces. Total stool activity was measured by emulsifying a known weight (0.3–1.0 Ogm) with 50–100 ml of water in a Waring blender. An aliquot of the emulsion (0.5–1 ml) was applied to a 1.25 inch platinum planchet. The planchets containing 3–10 mg of dry fecal residue were counted. The planchet count at infinite sample thinness was estimated using a standard self-absorption plot. The total radioactivity in individual daily stool samples was then calculated.

Radioactivity analysis, urine. An aliquot of each pooled 24-hour urine (0.5–1 ml) was planchetted for counting.

Counting was done in all instances on a gas flow, thin window proportional counter[1] with automatic sample changer. The total count was 10,000 or more per planchet. Correction for self-absorption was not done when crystalline material was counted as the amount of material on the planchet did not exceed 0.5 mg.

Crystalline stercobilin hydrochloride or porphyrin methyl ester (0.1–0.5 mg) in chloroform was applied to the planchet. The dry residue covered the surface of the planchet evenly after evaporation of the chloroform. After counting, stercobilin or porphyrin ester was dissolved from the planchets by repeated washing with small portions of chloroform. Essentially no colored material remained on the planchet after 2–3 washings. The pooled chloroform solution was quantitated by light absorption in an Evelyn colorimeter, using a 490 mμ and 400 mμ filter for stercobilin and porphyrin, respectively. The specific and total activity was calculated for each stool sample.

Results. Incorporation of ^{14}C into free and hemoglobin Proto- of the duck red cell hemolysate is shown in Table I. This is comparable to that reported previously (4) although in the present study relatively more ^{14}C was incorporated into hemoglobin Proto- (54%) and less into the free Proto- (10%) compared with 45% and 33%, respectively, in the previous study.

The porphyrin methyl ester crystallized from the feces, after hydrolysis, behaved entirely as 2-COOH porphyrin by paper chromatography (11). It was evident, however, that this was a mixture of Proto- and at least one other 2-COOH porphyrin, as in different samples the position of the Soret band maximum varied from 404–407 mμ, compared with 408 for authentic Proto- ester. The melting point of recrystallized porphyrin ester was 202 as compared with 231 for Proto- ester. On thin-layer chromatography (12) two spots were observed, one corresponding with Proto-, the other with deuteroporphyrin ester. The Soret band of the latter is at 399 mμ and a simple mixture of Proto- and deutero- (2:1) exhibits a single well defined absorption at 402 mμ rather than a double spectrum. Thus it is evident that the observed absorption is

[1] We are indebted to Dr. L. Zieve, Professor of Medicine, Minneapolis Veterans Hospital, for the use of the counter.

TABLE II. Recovery of Radioactivity in Fecal Proto- Group.

Day	mg/day	DPM $\times 10^3$/mg	Total DPM $\times 10^3$/day	% of administered ^{14}C
1	1.8154	11.9	21.60	0.15
2	2.8493	43.4	125.00	0.88
3	1.784	380.00	675.00	4.70
4	0.9785	975.0	950.00	6.70
5	1.2500	605.0	760.00	5.40
6	0.4920	256.0	126.00	0.89
7	0.4450	61.0	27.00	0.19
8	1.7010	19.0	32.0	0.23
9	—	—	—	—[a]
10–12	5.265	0.2	1.1	0.01
			DPM $\times 10^6$	
Total			2.717	19.2

[a] Stools not collected (loose B. M.).

compatible with a mixture containing a major proportion of Proto-. The possibility that the mixture also contained pemptoporphyrin (13) must be considered. This is a monovinyl, monodeuteroporphyrin (13) which has a Soret band at 405 mμ, mp 218°C. Pemptoporphyrin[2] has been found indistinguishable from Proto- with the Chu method of thin-layer chromatography. In any event the present porphyrin ester, believed to be at least mainly Proto-, behaved entirely as a 2-COOH porphyrin; any others which might be considered, such as pempto- or deutero-, are simply derivatives of Proto-. The term Proto- group is used in the following, in this sense. It is evident that the melting point of 202° may represent depression of a mixture of Proto- ester with smaller amounts of pempto- and/or deuteroporphyrin esters. This would be more in accord with the absorption maxima noted.

Table II includes the data on recovery of Proto-^{14}C in the feces. Radioactive Proto- group appeared in the stools as early as 12 hours after intraduodenal administration. Excretion in the feces continued for at least 8 days but activity had become negligible by day 10; peak activity was observed on day 4. Total Proto- group ^{14}C excreted in the feces accounted for 19.2% of the amount administered. The average total Proto- group excretion per day was approximately 1.5 mg, slightly higher than the usual normal range. The amount on day 2 was 2.85 mg. Radioactivity analysis (Table II) showed that Proto-^{14}C excretion on day 2 accounted for less than 1% of the administered (3.873 mg or 14.2 $\times$ 10^6 DPM) and therefore could not account for the apparent excess of total Proto- in the feces on that day. Newer information to be discussed elsewhere has made it clear that the normal range of variation has not yet been established with certainty and values considerably higher than this have at times been encountered in presumably normal individuals.

Stercobilin ^{14}C data are given in Table III. Excretion of radioactive stercobilin was detected on the second day (the earliest sample from which crystalline material could be obtained). Total activity in stercobilin accounted for approximately 1.5% of the administered Proto-^{14}C. The peak activity was on day 5 and the decline was slower than that observed with Proto-. The specific activity was unchanged on repeated recrystallization (5 $\times$).

Total activity in the stools of the first 12 days, as determined by direct planchetting (Table IV) accounted for 76% of the administered ^{14}C. Radioactivity in the Proto-

TABLE III. Recovery of Radioactivity in Stercobilin.[a]

Day	mg/day	DPM/mg	Total DPM $\times 10^3$/day
1	58.7	—	—[b]
2	92.7	180	16.7
3	123.2	400	49.3
4	115.1	317	36.5
5	228.8	256	58.6
6	148.8	126	18.7
7	94.0	—	—
8	495.0	31	15.3
9	—	—	—
10–12	280.0	27	7.6
Total			202.7

[a] Total activity in stercobilin was approximately 1.5% of administered.

[b] Crystalline material unavailable; activity not measured.

[2] Samples were kindly provided by Dr. J. M. French, St. Elizabeth Hospital, Birmingham, England, and by Dr. S. Sano, Kyoto University, Kyoto, Japan.

TABLE IV. Total Activity in Stools.

Day	Total DPM × 10^3	% of administered	Total DPM × 10^3 in Proto- group	Total DPM × 10^3 in stercobilin	Total DPM × 10^3 in other compounds
1	96.0	0.68	21.6	—	74.4[a]
2	330.0	2.3	125.0	16.7	188.3
3	3000.0	21.1	675.0	49.3	2275.7
4	3030.0	21.3	950.0	36.5	2043.5
5	3140.0	22.1	760.0	58.6	2321.4
6	900.0	6.35	126.0	18.7	755.3
7	240.0	1.69	27.0	—	213.0[a]
8	30.0	0.21	32.0	15.3	— [c]
9	—	—	—	—	— [b]
10–12		0.0	1.1	7.6	— [c]
	DPM × 10^6		DPM × 10^6	DPM × 10^6	DPM × 10^6
Total	10.77	76.0	2.717	0.203	7.872

[a] Activity in stercobilin is included with this fraction on that day.

[b] Stools not collected.

[c] Total activity in stools too small to be determined accurately by direct planchetting.

and stercobilin fractions accounted for only 20.7%. The difference must relate to unidentified compounds in the feces, either derivatives of Proto- or bile pigment not included in the pure samples analyzed. Approximately 24% of the administered radioactivity was unaccounted for in the feces or the urine during the collection period.

Only minimal amounts of radioactivity were found in the urine in the first 3 days.

Discussion. The present study provides evidence for an enterohepatic circulation (EHC) of Proto-. The early appearance of both Proto- group ^{14}C and stercobilin ^{14}C in the feces points to the absorption of at least a fraction of the administered Proto- and its conversion in the body to bile pigment. Labeling of fecal Proto- group had an earlier peak (four day) and a more rapid fall off than that of stercobilin (peak on fifth day) which is in accord with a precursor relationship. It was shown in recent studies that protoporphyrin is converted in part to bilirubin in the liver, probably via an hepatic heme (4, 14).

The possibility might be considered that bile pigment was formed from the labeled protoporphyrin by the fecal flora, although no evidence of such a conversion has been found in the past (15). Although we have not investigated this in any detail, a recent experiment may be mentioned briefly in which ^{14}C labeled protoporphyrin (1.75×10^6 DPM) was incubated with normal feces under nitrogen for 24 hours, after which the stercobilin was isolated. After repeated recrystallization no radioactivity was detectable.

If a nonabsorbable substance is introduced in the gastrointestinal tract of a subject with normal bowel habits, one would anticipate that most of it would be excreted in 48–72 hours. As seen in Table II the peak value was on day 4 and the amount was almost as great on day 5, which suggests an enterohepatic circulation. Berlin (16) noted continued excretion of highly labeled Proto- in the stools of a human subject for 3 days following administration of ALA-^{14}C and on this basis concluded that Proto- underwent an enterohepatic circulation. The prolonged excretion of Proto-^{14}C in the feces in the present study (throughout the entire collection period) indicates some degree of continuous recirculation. It is probable that with each circulation a small fraction of the absorbed Proto-^{14}C is converted to bile pigment, another is eliminated in the feces as unaltered Proto-, or as immediate derivatives such as deutero- or pemptoporphyrin formed by the reducing activity of the intestinal flora, and a third is degraded to other compounds. Degradation probably occurs in the liver and/or in the intestine, possibly through bacterial activity.

These degradation products contribute to the total radioactivity in the feces.

Total stool activity accounted for 76% of the administered Proto-^{14}C. The balance was unaccounted for. Incorporation in circulating hemoglobin was not examined but in view of the known minimal incorporation in other studies (4, 17) this may be assumed to have been insignificant.

Summary and Conclusions. Proto-^{14}C was administered through a duodenal tube in a normal subject. Serial isolations of crystalline 2-COOH porphyrin and stercobilin from the feces during the next 12 days, and radioactivity measurements provided evidence that some fraction of the administered Proto- underwent an enterohepatic circulation. A small proportion (1.5–3%) was converted to bile pigment. The data point to a continuous partial enterohepatic circulation of Proto- with loss of a constant fraction on each circulation.

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Age Changes in Body Weight and in Several Blood Componets of Conventional versus Miniature Pigs (32830)

W. G. POND, R. J. BANIS, L. D. VAN VLECK, E. F. WALKER, JR., AND P. CHAPMAN
(Introduced by J. K. Loosli)
Department of Animal Science, Cornell University, Ithaca, New York

The utility of the pig in biomedical research has been recognized for some years (1) but its normal mature weight of 250–350 kg constitutes a disadvantage in convenience and economy. The responsiveness of the pig to selection for small size (2) has been applied to the development of several strains of "miniature" pigs for use as research animals (3–7). The safe extrapolation of physiological data obtained with the miniature pig to the conventional pig or to man rests on the assumption that important changes in biochemical and physiological parameters have not accompanied the change in mature size accomplished by selection. A considerable body of hematological and physiological data have been accumulated for the miniature pig (8–11). However, few direct comparisons have been made of the miniature and conventional pig kept under similar environmental conditions. The data reported herein were obtained to provide comparative information on