- Mandelstam, J. (1957). *Nature, Lond.*, **179**, 1179.
 Mandelstam, J. (1958). *Biochem. J.* **69**, 110.
 Mandelstam, J. & Halvorsen, H. (1960). *Biochim. biophys. Acta*, **40**, 43.
 Morse, M. L. & Carter, C. E. (1949). *J. Bact.* **58**, 317.
 Postgate, J. R., Crumpton, J. E. & Hunter, J. R. (1961). *J. gen. Microbiol.* **24**, 15.
 Rotman, B. (1958). *J. Bact.* **76**, 1.
 Schachman, H. K., Pardee, A. B. & Stanier, R. Y. (1952). *Arch. Biochem. Biophys.* **38**, 245.
 Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.
 Stickland, L. H. (1951). *J. gen. Microbiol.* **5**, 698.
 Strange, R. E. (1961). *Nature, Lond.*, **191**, 1272.
 Strange, R. E., Dark, F. A. & Ness, A. G. (1961). *J. gen. Microbiol.* **25**, 61.
 Tissieres, A., Watson, J. D., Schlessinger, D. & Hollingworth, B. R. (1959). *J. molec. Biol.* **1**, 221.
 Tracey, M. V. (1952). *Biochem. J.* **52**, 265.
 Wade, H. E. (1960). *Biochem. J.* **77**, 534.
 Wade, H. E. (1961). *Biochem. J.* **78**, 457.
 Wade, H. E. & Lovett, S. (1961). *Biochem. J.* **81**, 319.
 Wade, H. E. & Morgan, D. M. (1957). *Biochem. J.* **65**, 321.
 Yemm, E. W. & Cocking, E. C. (1955). *Analyst*, **80**, 209.

Biochem. J. (1963) **86**, 203

Excretion of Various Porphyrins and their Corresponding Porphyrinogens by Rabbits after Intravenous Injection

By SEIYO SANO* AND C. RIMINGTON

Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

(Received 8 August 1962)

Numerous investigations have been conducted on the fate of various porphyrins after ingestion or injection without an intermediary metabolism of porphyrins having been conclusively established. Recent biochemical evidence shows that the actual intermediates in the biosynthesis of haem are not porphyrins, however, but their reduction products, the porphyrinogens, derivable from hexahydroporphin. The chemistry of these readily autoxidizable substances has been studied, but their behaviour and metabolism *in vivo* is still unknown. That they occur *in vivo* is certain but several questions remain unanswered, e.g.: (1) By what mechanism are the tetrapyrrole pigments kept *in vivo* in the reduced form as porphyrinogens? (2) Do porphyrinogens combine with proteins? (3) Is any protoporphyrinogen found *in vivo*? (4) Do porphyrins normally occur *in vivo*? (5) If so, at what stage and in what site does the oxidation of porphyrinogen to porphyrin occur? (6) What factors control the urinary excretion of porphyrins and porphyrinogens; is there a relation to the number of carboxylic functions in the molecule?

That a considerable fraction of the urinary coproporphyrin ('urospectrine') existed in fresh specimens as a colourless precursor was reported by Sallet (1896); but this observation was neglected until Watson, de Mello, Schwartz, Hawkinson & Bossenmaier (1951) showed that the non-fluorescent chromogen was in many respects similar to chemically prepared coproporphyrinogen and was

readily oxidized to coproporphyrin by dilute iodine. In fresh urines, 25–66% of the coproporphyrin was present as precursor. Holeček (1957*a*) found that with appropriate precautions 97% of the total could be identified as porphyrinogen. Weatherall (1952) found in rabbits that injected coproporphyrin III was eliminated in the bile and not the urine, and suggested that in lead-poisoned animals it was entirely coproporphyrinogen that gave rise to the high urinary coproporphyrin concentrations found by the usual methods of determination. Hoffbauer, Watson & Schwartz (1953) showed that coproporphyrin injected into the normal rat did not appear in the urine whereas with animals poisoned by carbon tetrachloride a proportion of the dose was so eliminated. Using rabbits, Heikel, Knight, Rimington, Ritchie & Williams (1960) compared the behaviour of intravenously injected coproporphyrinogen III and coproporphyrin III. Both were eliminated in part in the fistula bile, but, whereas coproporphyrinogen passed readily into the urine, no such elimination of injected coproporphyrin occurred.

The present paper describes further studies on the excretion of various porphyrins and porphyrinogens after their intravenous injection into rabbits.

EXPERIMENTAL

Animals

Male adult rabbits weighing 2–2.5 kg. were used. An indwelling urinary catheter was inserted and urine was collected for analysis every 2 hr. after the intravenous

* Present address: Department of Public Health, Faculty of Medicine, Kyoto University, Kyoto, Japan.

injection of porphyrin or porphyrinogen solution. After 6–8 hr. the catheter was removed and the subsequent samples were collected by spontaneous urination or further catheterization. Rabbits with external biliary fistulae were kindly prepared by Dr T. A. J. Heikel.

Materials

Porphyrins. Uroporphyrin III octamethyl ester, m.p. 264°, prepared from turacin (Nicholas & Rimington, 1952), was hydrolysed by 6N-HCl for 1 day at room temperature in the dark. The acid was evaporated under reduced pressure, the material further dried over NaOH pellets *in vacuo*, and the porphyrin dissolved in 0.01N-KOH to make a stock solution of 1 mM. The concentration was checked spectrophotometrically in N-HCl at 405 m μ (ϵ 50 $\times$ 10⁴) or 553 m μ (ϵ 1.75 $\times$ 10⁴) (Mauzerall & Granick, 1958).

Coproporphyrin III tetramethyl ester, initial m.p. 166°, remelt 180°, was derived from diphtheria-toxoid broth (Gray & Holt, 1948). The ester was hydrolysed with 6N-HCl as for uroporphyrin and a 1 mM stock solution was prepared in the same way. The concentration was checked in N-HCl at 401 m μ (ϵ 47 $\times$ 10⁴) or at 549 m μ (ϵ 1.75 $\times$ 10⁴) (Mauzerall & Granick, 1958).

Protoporphyrin IX was prepared by Fischer's (1953) method. No contamination by mesoporphyrin was observed.

Deuteroporphyrin IX was prepared as described by Fischer & Orth (1943).

One sample of haematoporphyrin hydrochloride was kindly supplied by Dr G. Y. Kennedy of Sheffield; a second sample was a specimen from Japan.

Mesoporphyrin IX was prepared by the reduction of protoporphyrin IX with HI as described by Fischer & Orth (1943).

For intravenous injection, the requisite quantity of each porphyrin solution was neutralized and diluted to 20 ml. with 67 mM-sodium phosphate buffer containing 0.9% (w/v) of NaCl. The final concentration of porphyrin solution was approx. 0.15–0.2 mM.

Porphyrinogens. Uroporphyrinogen III was prepared by the reduction of 3 ml. of 1 mM-uroporphyrin solution (containing 2.52 mg. of uroporphyrin III) with freshly ground 3% (w/w) sodium amalgam under nitrogen in dim red light. Under the same illumination, the mixture was filtered on a sintered-glass filter which was washed with water; the filtrate plus washings were adjusted to pH 7.2 with a saturated solution of sodium dihydrogen phosphate. An equal volume of 1.8% (w/v) NaCl was added and the mixture was diluted to 20 ml. with 0.9% (w/v) NaCl solution through which N₂ had been bubbled. Fluorescence of this preparation was negligible. The solution was administered slowly through the ear vein of the rabbit under dim red light. Coproporphyrinogen solutions were prepared by the same method for injection. Protoporphyrin (2 mg.) was dissolved in 0.02N-KOH and 2–3 drops of ethanol were added to increase its solubility. Protoporphyrinogen was prepared from this solution by reduction with sodium amalgam for 2 min. at 80° (Sano & Granick, 1961). If foaming occurred during reduction, more ethanol was added. Haematoporphyrinogen was prepared by a 2 min. reduction of haematoporphyrin in the same way with sodium amalgam. No mesoporphyrinogen was formed during the reduction. Mesoporphyrinogen was prepared from mesoporphyrin with sodium amalgam in a

manner similar to uroporphyrinogen. The addition of digitonin (3.2 mg.) facilitated this preparation. Deuteroporphyrinogen was prepared from deuteroporphyrin with sodium amalgam in a manner similar to uroporphyrinogen.

Analysis of porphyrins

Ether and ethyl acetate were freshly distilled and free from peroxides. Oxidation of porphyrinogens was accomplished by treatment with dilute iodine, except for protoporphyrinogen. Protoporphyrinogen was oxidized in ethyl acetate-acetic acid solution exposed to mild illumination (Sano & Granick, 1961).

Porphyrins in urine and faeces were determined by a modification of Rimington's (1961*a*) method. Coproporphyrin in urine was extracted with ethyl acetate-acetic acid (3:1, v/v). The extract was washed several times with 3% (w/v) sodium acetate and once with aqueous 0.005% (w/v) iodine, and then an equal volume of ether was added to the ethyl acetate. Coproporphyrin was removed from this mixture by extracting three or four times with 2.5N-HCl until no fluorescence remained in the upper layer. The aqueous acid solution was adjusted to pH 3.5 with a saturated solution of sodium acetate and extracted into 15 ml. of ether. The ether was washed three times with water. Coproporphyrin was extracted with three 3 ml. portions of 0.1N-HCl and the solution was diluted to 10 ml. with ethanol for spectrophotometry. The amount of coproporphyrin was calculated by using the correction formula of Rimington & Sveinsson (1950) or directly from the extinction at 401 m μ (ϵ 47 $\times$ 10⁴). The latter procedure gave a slightly higher value than the former.

Uroporphyrin in urine was determined by the method of cyclohexanone extraction (Rimington, 1961*a*), and the correction formula of Rimington & Sveinsson (1950) was used for the calculation. Protoporphyrin was sought by extracting the urine by the same method as for coproporphyrin, without the use of iodine, and then removing any further porphyrin from the ether with 1.5N-HCl; paper chromatography with lutidine-ammonia served for identification. The extinction was measured at the region of the Soret band for protoporphyrin (408 m μ ; ϵ 24 $\times$ 10⁴). Mesoporphyrin, deuteroporphyrin and haematoporphyrin were determined in the same way as was coproporphyrin and each porphyrin was further characterized by paper chromatography with lutidine-ammonia (Falk & Benson, 1953) or kerosene-chloroform (Bogorad & Granick, 1953).

Analysis of faecal porphyrin is rendered difficult by the presence of chlorophyll derivatives and bile pigments. A sample (5 g.) of faeces was homogenized with 50 ml. of ethyl acetate-acetic acid (3:1, v/v), the mixture filtered through a sintered-glass filter, and the residue extracted repeatedly with this solvent until free from porphyrin. The combined filtrates were washed four times with 50 ml. of 3% (w/v) sodium acetate and once with 0.005% (w/v) I₂, and the aqueous solution was discarded except in the uroporphyrin experiments. An equal volume of ether was added to the ethyl acetate and the porphyrins were extracted with 2.5N-HCl. The acid solution was made neutral to Congo red with sodium acetate and the porphyrins were extracted into ether. From this solvent they were extracted in two fractions, with 0.1N- and 1.5N-HCl respectively. The 0.1N-HCl extract was shaken with chloroform to remove any deuteroporphyrin or phylloerythrin (Heikel *et al.* 1960). The remaining acid solution

showed a maximum at 401 $m\mu$ and Rimington's (1961*a*) correction formula was used. Protoporphyrin was highly contaminated by phylloerythrin in the 1.5 *N*-HCl fraction which showed Soret maxima at 410 and 420 $m\mu$ respectively and heterogeneity by lutidine-ammonia paper chromatography. Rimington's (1961*a*) correction formula gave a satisfactorily accurate value for protoporphyrin.

Determination of porphyrinogen and porphyrin, especially coproporphyrinogen and protoporphyrin, in the bile was performed as follows. To 2.7 ml. of bile, freshly collected with the exclusion of light and under a slow stream of N_2 bubbles, 0.4 ml. of 3*M*- $CaCl_2$ and 0.3 ml. of 6*N*-NaOH were added. The mixture was stirred well with a glass rod, and the precipitate was rapidly collected by centrifuging in the dark. A separate trial on 65.4 μ g. of coproporphyrinogen, added to 2.5 ml. of bile, showed that 75% of the coproporphyrinogen remained in the supernatant while coproporphyrin was found in the precipitate contaminated by some coproporphyrinogen. When coproporphyrin (65 μ g.) was added in place of coproporphyrinogen, adsorption on the $Ca(OH)_2$ was complete. After being washed with a small amount of 0.1 *N*-NaOH, the pale-green sediment was suspended in ethyl acetate-acetic acid (3:1, v/v) and exposed to gentle illumination to oxidize any porphyrinogen to porphyrin before extraction with HCl in the usual way. The supernatant was mixed with ethyl acetate-acetic acid mixture and treated similarly.

RESULTS

Uroporphyrin III and uroporphyrinogen III. After intravenous injection of uroporphyrin III (2.52 mg. in 20 ml.), rapid urinary excretion was observed. The urine collected by catheter had a chocolate-brown colour and fluoresced strongly under ultraviolet light. Within 2 hr., 1.03 mg. of uroporphyrin was recovered in 20 ml. of urine (Table 1) and the total recovery in 24 hr. was 70% of the dose. No uroporphyrin was detected in the faeces.

Uroporphyrinogen III was more rapidly excreted in the urine than was uroporphyrin III. Fresh urine had a yellow colour and showed no fluorescence. Fluorescence appeared promptly on the addition of iodine. Within 2 hr., 1.64 mg. of uroporphyrinogen was excreted in 26 ml. of urine. The total recovery from the urine was 67% of the dose. There was no increase of coproporphyrinogen.

Fate of injected coproporphyrinogen III. Coproporphyrinogen III administered intravenously to a rabbit was excreted in part as coproporphyrinogen in the urine. The total recovery by this route was 4-6% of the dose within 24 hr. Faecal excretion accounted for 11%. After injection of coproporphyrinogen III there was also a rise in the faecal content of protoporphyrin, denoting a conversion of coproporphyrinogen into this pigment which accounted for 25-30% of the dose (Table 2).

Effect of reducing agents. It is well known that sulphhydryl compounds such as thioglycollate or ascorbic acid prevent the autoxidation of a porphyrinogen. Although the coproporphyrinogen solutions prepared by the procedure described above showed very little fluorescence, it is likely that they might be in part oxidized *in vivo* after administration. Thioglycollate (75 mM) or ascorbic acid (200 mg.) was therefore added to a coproporphyrinogen solution after its preparation and before injection. A two- to three-fold increase of coproporphyrinogen excretion in the urine was observed (Table 3). In another rabbit, 200 mg. of ascorbic acid was injected subcutaneously each day for 6 days (total amount 1.2 g.) before the intravenous administration of coproporphyrinogen III. The highest excretion of urinary coproporphyrinogen (12.7%) was observed in this animal (Table 3).

Table 1. *Urinary excretion of intravenously injected uroporphyrin III and uroporphyrinogen III by a normal rabbit*

Experimental details are given in the text. The samples obtained 0-2 hr. after injection were assayed by diluting 1 ml. of urine to 100 ml. with 0.1 *N*-HCl, exposing this solution to light in the test for uroporphyrinogen, recording the extinction at 406 $m\mu$, 430 $m\mu$ and 380 $m\mu$, and applying the correction formula of Rimington & Sveinsson (1950). Other samples were analysed by the standard method involving extraction (Rimington, 1961*a*).

Day	Substance injected	Time after injection (hr.)	Vol. (ml.)	Uroporphyrin (μ g.)	Uroporphyrinogen (μ g.)	Coproporphyrinogen (μ g.)
1	Before injection	-2-0	23.0	Trace	—	—
	Uroporphyrin (2.52 mg.)	0-2	20.0	1030	—	—
		2-4	9.5	581	—	—
		4-6	4.0	117	—	—
		6-18	33.0	32	—	—
		18-24	35.0	9.8	—	—
4	Before injection	-2-0	33.0	—	Trace	—
	Uroporphyrinogen (2.5 mg.)	0-2	26.0	—	1640	Trace
		2-4	4.6	—	19	Trace
		4-6	10.0	—	5	Trace
		6-24	145.0	—	2	Trace

Metabolism of coproporphyrinogen III in the liver. A rabbit with an external biliary fistula was prepared for this experiment. Coproporphyrinogen III solution was injected intravenously 30 min. after the operation. Bile was collected into a centrifuge tube completely shielded from light and under a stream of nitrogen bubbles. The bile collected during each 0.5 hr. was analysed for porphyrin and porphyrinogen by the method described above. As shown in Table 4, injected coproporphyrinogen was excreted in the bile within 30 min.; subsequently the amount decreased rapidly. After 30 min. a rise in the amount of protoporphyrin was

observed which was sustained for many hours. This result demonstrates the conversion of coproporphyrinogen into protoporphyrin *in vivo* as was observed by Sano & Granick (1961) to occur in liver mitochondria *in vitro*. Protoporphyrinogen was not observed except for a trace in the bile collected during the first 30 min. (Table 4).

Effect of injury caused by carbon tetrachloride. A 20% (w/w) solution of carbon tetrachloride in olive oil was prepared and 0.5 ml./kg. was injected subcutaneously into a rabbit according to the time schedule shown in Table 5. There was only a slight, if any, increase in total urinary copropor-

Table 2. *Excretion of intravenously injected coproporphyrinogen III by normal rabbits*

Experimental details are given in the text.

Rabbit	Substance injected	Time after injection (hr.)	Urine		Faeces		
			Vol. (ml.)	Coproporphyrinogen (μ g.)	Wt. (g.)	Coproporphyrin (μ g./day)	Protoporphyrin (μ g./day)
A	Before injection	-24-0	40.0	9.5	—	—	—
	Coproporphyrinogen III (2.2 mg.)	0-2	43.0	82.0	—	—	—
		2-4	9.0	8.2	—	—	—
		4-6	97.0	3.0	—	—	—
		6-24	90.0	14.0	—	—	—
B	Before injection	-24-0	100.0	5.6	45.2	96.7	377.4
	Coproporphyrinogen III (2.2 mg.)	0-3	114.5	79.6	55.5	202.6	763.0
		3-5	11.0	2.2			
		5-7	25.0	5.7			
		7-24	105	9.2	59.0	132.0	401.2
		24-48	125	4.8			

Table 3. *Effect of reducing agents on the urinary excretion of intravenously injected coproporphyrinogen III by normal rabbits*

Experimental details are given in the text.

Rabbit	Substance injected	Time after injection (hr.)	Vol. (ml.)	Coproporphyrinogen III (μ g.)
C	Before injection	-24-0	287	6.4
	Coproporphyrinogen (2.5 mg.) + potassium thioglycollate (75 mm)	0-2	76	235.5
		2-4	9	20.1
		4-6	22	5.0
		6-24	470	9.2
		24-48	270	9.5
D	Before injection	-24-0	140	9.1
	Coproporphyrinogen (2.5 mg.) + ascorbic acid (200 mg.)	0-2	20	171.2
		2-4	20	35.8
		4-6	13	4.7
		6-24	103	1.3
		24-48	130	6.6
E	Ascorbic acid (200 mg.) subcutaneously injected daily for 6 days			
	Before injection	-24-0	124	14.0
	Coproporphyrinogen (2.26 mg.)	0-2	24	243.7
		2-4	8	24.7
		4-6	7	8.1
		6-24	45	8.7
		24-48	89	30.0

phyrin after two such doses. On day 12, 2.2 mg. of coproporphyrinogen III was injected intravenously, of which dose 133 μ g. (6%) appeared as coproporphyrinogen in the urine. This is slightly higher than expected for a normal animal. Carbon

tetrachloride was administered again on day 14, and on the next day 2.2 mg. of coproporphyrin III was given intravenously in the usual way. A total of 69.4 μ g. appeared in the urine in 24 hr. (47.0 μ g. in the first 2 hr.). The procedure of this experiment

Table 4. *Metabolism of injected coproporphyrinogen III in liver by a rabbit with external biliary fistula*

Experimental details are given in the text. N.D., Not determined.

Substance injected	Time after injection (min.)	Vol. (ml.)	Bile			
			In $\text{Ca}(\text{OH})_2$ supernatant		In $\text{Ca}(\text{OH})_2$ precipitate	
			Copro- porphyrinogen (μ g.)	Protopor- phyrinogen (μ g.)	Copro- porphyrin (μ g.)	Protopor- phyrin (μ g.)
Before injection	-60 to -30	4	N.D.	N.D.	N.D.	0.8
	-30-0	4	N.D.	N.D.	0	1.3
Coproporphyrinogen (2.34 mg.)	0-30	2.5	93.3	3.9	76.2	12.5
	30-60	2.5	7.4	0	9.7	66.0
	60-90	3.0	1.3	0	4.8	48.8
	90-120	2.5	0.7	0	2.9	27.5
	120-180	5.1	0.6	0	2.2	43.7
	180-300	7.5	0.5	0	2.0	43.3
	300-480	12.0	0.5	0	1.5	50.0
	480-1440	48.0	0	0	Trace	22.0

Table 5. *Urinary excretion of intravenously injected coproporphyrin III and coproporphyrinogen III by a rabbit (wt. 3 kg.) poisoned with carbon tetrachloride*

Experimental details are given in the text.

Day	Substance injected	Copro- phyrinogen III or copro- porphyrin III (μ g./day)			
	Before injection	6.5			
		8.3			
		9.0			
1	CCl ₄ (0.3 g.)	2.9			
2		13.6			
3		30.6			
4		21.3			
5					
6		13.4			
7		26.9			
8		10.7			
9		4.9			
10		2.6			
11		20.7			
12	Coproporphyrinogen III (2.2 mg.)	133.0			
13		7.5	Time	Vol.	Coproporphyrin
14	CCl ₄ (0.3 g.)	2.0	(hr.)	(ml.)	(μ g.)
			0-2	51.5	47.0
			2-4	6.0	6.9
15	Coproporphyrin (2.2 mg.)	69.4	4-6	1.7	1.7
			6-24	62.5	13.8
26		16.9			
27		18.8	Time	Vol.	Coproporphyrin
28	CCl ₄ (0.3 g.)	12.2	(hr.)	(ml.)	(μ g.)
			0-2	8	49.3
29	Coproporphyrin (2.2 mg.)	67.0	2-4	3.5	13.9
			4-6	25	3.8
			Died		

was repeated on days 28 and 29 when the 24 hr. urine contained 67 μ g. (49.3 μ g. in the first 2 hr.).

To examine whether the excretion was as coproporphyrin or coproporphyrinogen, the following experiments were performed. To fresh catheter urine, which already exhibited a slight pink fluorescence, dilute iodine at pH 7 was added: there was no increase in fluorescence. Another 2 hr. sample of urine was adjusted to pH 3.5 by formate buffer (no increase of fluorescence) and porphyrin was then rapidly extracted into ether. The ether layer exhibited fluorescence and four absorption bands visible in the hand spectroscope. The addition of iodine failed to increase the fluorescence. There is little doubt, therefore, that the porphyrin excreted under these circumstances was indeed coproporphyrin III and not the corresponding porphyrinogen. After liver injury of a degree caused by this treatment with carbon tetrachloride, there is no evidence of reduction to coproporphyrinogen III of coproporphyrin III administered intravenously. The appearance in the urine of some of the injected coproporphyrin may be due to hepatic or renal damage (enhanced permeability of the glomerulus), or to both, caused by the toxic agent.

Effect of ligation of the common bile duct. Two rabbits were prepared by surgical ligation of the common bile duct, the cystic duct being left patent, followed by catheterization. They were kept in warm surroundings (30°) and 15 min. after operation a solution of coproporphyrin III (2 mg. in 20 ml.) was injected intravenously into each over the course of 15 min. Excretion of coproporphyrin in the urine was observed in both animals as shown in Table 6. Excretion rose during the first 4 hr. and then remained at a high level until death. The total urinary excretion accounted for 66.7 and 49.8% respectively in the two experiments, peak eliminations reaching 798 μ g./100 ml. in the first rabbit, which had a preinjection base-line excretion of 3 μ g./100 ml., but reaching 1470 μ g./100 ml. in the second rabbit in which the basal excretion was 10.2 μ g./100 ml. Careful examination showed that the pigment excreted was indeed coproporphyrin III and not the corresponding porphyrinogen. Post-mortem analysis accounted for 3.2–6.7% of the dose in the livers and 0.16–1.7% in the kidneys of the two rabbits used.

Two explanations of these results seemed possible, namely: (a) that constituents of the bile entering the circulation after ligation of the common bile duct increase the permeability of the renal apparatus, by injury or otherwise, thus allowing coproporphyrin to pass into the urine, such an effect being fully developed after 4 hr.; (b) injected coproporphyrin is concentrated avidly by the liver cells but, as no biliary secretion is

possible, the pigment diffuses back into the circulation, reaches the kidney and is excreted. Such release would have to gain maximum momentum after about 4 hr.

Into another rabbit, prepared by ligation of the bile duct, either protoporphyrinogen or haematoporphyrin was injected but neither appeared in the urine.

In control experiments, from about 2 hr. after ligation of the common bile duct, bilirubin was detectable in the urine, and after about 4 hr. haematuria was observed, indicating injury to the kidney by constituents of the bile.

To elucidate the situation further, blood concentrations of coproporphyrin or coproporphyrinogen were determined at suitable intervals of time in rabbits with ligated common bile ducts and to which coproporphyrin or coproporphyrinogen had been administered not more than 15 min. after establishment of the ligature. These values were compared with the concentrations found in control animals receiving similar injections but without interference to the biliary excretory system. Blood samples (2–3 ml.) were taken from the ear vein and extracted with ethyl acetate–acetic acid (3:1, v/v). Coproporphyrinogen was allowed to oxidize to coproporphyrin in this mixture over-

Table 6. *Excretion of coproporphyrin in the urine of the rabbit with ligated common bile duct after intravenous injection of coproporphyrin III*

Experimental details are given in the text. Coproporphyrin III (2 mg.) was injected at 0 hr. At death, the liver (92 g.) and kidneys (13.8 g.) of rabbit A contained 63.7 and 3.2 μ g. of coproporphyrin III respectively; the liver (60 g.) and kidneys (16 g.) of rabbit B contained 133.0 and 33.8 μ g. respectively.

Rabbit	Time (hr.)	Vol. (ml.)	Coproporphyrin	
			Total (μ g.)	Concn. (μ g./100 ml.)
H	–24–0	318	9.4	3
	0–2	28	26.8	96
	2–4	0	0	0
	4–8	44*	182.3	414.3
	8–11	12.5	45.3	362.4
	11–15	19	127.6	671.6
	15–24	21.5	171.6	798.1
	24–48	58	432.2	745.2
	48–72	63	361.5	573.8
	72–96	27	55.1	204.1
J	–24–0	183	18.7	10.2
	0–2	59	40.3	68.3
	2–4	0	0	0
	4–8	20*	294.0	1470.0
	8–11	13	160.7	1236.2
	11–24	30	426.1	1420.3
	24–30	6	74.6	1243.3
	Died			

* This and subsequent urines were deep brown.

Fig. 1. Blood concentrations of coproporphyrin (○) or coproporphyrinogen (■) after intravenous injection of these materials (2 mg.) into normal rabbits (—) or rabbits with surgical ligature of the common bile duct (···).

Table 7. Blood and urinary coproporphyrin concentrations in a rabbit with ligated common bile duct after the intravenous injection of coproporphyrin III

Experimental details are given in the text. Coproporphyrin III (2 mg.) was injected 15 min. after the operation.

Time after injection (min.)	Concn. of coproporphyrin (μg./100 ml.)	
	In blood	In urine
0	3.8	120
10	300.0	
30	178.2	
60	233.7	
90	—	874
120	352.9	1258
240	301.7	993
480	390.0	483
600	373.3	1700

night; the subsequent procedure followed standard methods.

The results are shown in Fig. 1. Unfortunately, the 10 min. sample after injection of coproporphyrinogen into the normal rabbit was lost by breakage. It is evident that both coproporphyrin and coproporphyrinogen disappear very rapidly from the blood stream after injection, presumably owing principally to avid uptake by the liver. In the case, however, where biliary excretion was arrested by ligation of the common bile duct, there was a fairly prompt return of coproporphyrin to the circulation, coinciding roughly with the commencement of bilirubinuria, and continuing at a gradually increasing level over a long period of time. Table 7 records the excretion of coproporphyrin in the urine together with the blood porphyrin concentrations attained in such an animal.

Effect of lead poisoning. Lead acetate solution (0.742%, w/v) was injected subcutaneously into a rabbit in a daily dose of 10 mg. of lead/kg. There was a significant elevation of the concentration of urinary coproporphyrin as a result of the poisoning.

When coproporphyrinogen III (2.3 mg.) was injected intravenously on day 8, there was considerable excretion of coproporphyrinogen in urine but the percentage of the dose was not so high as with the combination of a reducing agent with coproporphyrinogen III. Injected coproporphyrin III was not excreted in the urine (Table 8).

Fate of injected protoporphyrinogen. Protoporphyrinogen solution (2.2 mg. in 20 ml.) was injected intravenously. Only a trace of protoporphyrinogen was excreted in the urine, and 0.6 mg. of protoporphyrin was recovered from the faeces. Attempts to inject a protoporphyrin solution were abandoned since it became colloidal at neutral pH.

Fate of injected deuteroporphyrin, deuteroporphyrinogen, mesoporphyrin and mesoporphyrinogen. No urinary excretion was detected of injected mesoporphyrin or mesoporphyrinogen. Similarly, deuteroporphyrin was not excreted, but a trace of deuteroporphyrinogen was found in the urine after its intravenous injection.

Fate of injected haematoporphyrin and haematoporphyrinogen. Haematoporphyrin (2.5 mg. in 20 ml.) solution was administered intravenously. Only 12 μg. was recovered from the urine in 24 hr. and none subsequently. About 0.6 mg. of haematoporphyrin was found in the faeces within a period of 7 days. Injected haematoporphyrinogen also failed to appear in more than traces in the urine; only 10.5 μg. of haematoporphyrinogen was excreted in 24 hr. In one case haematoporphyrin was excreted as a metal complex. After the metal was removed by concentrated sulphuric acid, 79 μg. haematoporphyrin was obtained.

DISCUSSION

These studies support the hypothesis that the excretion of porphyrin by the kidney is influenced by the number of carboxyl groups in the porphyrin molecule. Thus the octacarboxylic uroporphyrin III was excreted exclusively in the urine, and the tetracarboxylic coproporphyrin III and all the dicarboxylic porphyrins examined were excreted only in the faeces by way of the bile. With the porphyrinogens, a similar influence seems to be exerted by the number of carboxylic functions, but no dicarboxylic porphyrinogens entered the urine in more than trace amounts. Whether this selectivity is a property solely of the renal barrier or results in part from the action of the liver in concentrating porphyrins or porphyrinogens from the blood stream was further elucidated by the experiment in which blood concentrations and urinary excretion were followed in animals with surgical ligation of the common bile duct. As shown in Fig. 1, there is a very active uptake of coproporphyrin and a somewhat less active uptake of copro-

Table 8. *Urinary excretion of intravenously injected coproporphyrinogen III and coproporphyrin III by a rabbit (wt. 3 kg.) with lead poisoning*

Experimental details are given in the text.

Day	Substance injected	Coproporphyrinogen III ($\mu\text{g./day}$)			
	Before injection	11.0			
		7.4			
		14.7			
		5.5			
1	Pb (20 mg.)	9.1			
2		38.9			
3		95.7			
4		71.1			
5		50.0			
6		59.7			
7		47.7			
8	Coproporphyrinogen III (2.3 mg.)	184.4	Time (hr.)	Vol. (ml.)	Coproporphyrinogen ($\mu\text{g.}$)
			0-2	25.0	157.0
			2-4	7.5	11.0
			4-10	16.0	6.1
			10-24	33.0	10.3
9		18.9			
10		56.0			
11		50.0			
12		109.0			
13		73.0			
14		143.6	Time (hr.)	Vol. (ml.)	Coproporphyrinogen ($\mu\text{g.}$)
15	Coproporphyrin III (2.3 mg.)	50.1	0-2	16.0	19.7
			2-4	14.0	19.2
			4-6	27.5	6.7
			6-24	27.0	4.5

porphyrinogen resulting in rapid diminution of the blood concentrations of these materials after injection. If, however, biliary excretion is prevented by ligature, coproporphyrin returns to the circulation in increasing concentration. Coincident with this, urinary excretion increases. It seems evident, therefore, that the hepatic concentrating mechanism must also play a significant role in determining whether or not porphyrin is excreted in the urine. A. J. M. Reese & C. Rimington (unpublished work) have demonstrated that hepatic cells show no avidity for injected uroporphyrin, even in the nephrectomized animals. This may be a factor contributory to the rapid renal excretion of injected uroporphyrin.

Still further evidence may be gathered from the effects of pathological disturbance. Heikel *et al.* (1960) observed that, in the rabbit poisoned with icterogenin, the injection of coproporphyrin III was followed by its excretion in the urine; icterogenin inhibits biliary secretion. Urinary excretion similarly occurs in the animal poisoned with carbon tetrachloride. The possibility cannot be overlooked, however, that the renal cells may be injured and their permeability to porphyrins thereby altered, either by direct action of these toxic agents or by the constituents of the bile

gaining access to the circulation during jaundice. Rabbits with ligated bile ducts showed both bilirubinaemia and haematuria.

We think that the excretion of urinary porphyrin is probably governed largely by the capacity of the liver to concentrate pigment from the blood stream, but it may also be influenced by damage to the renal apparatus.

Fischer (1915-1916) found that uroporphyrin I (from Petry urine) taken by mouth is excreted unchanged in the stool and that parenteral injection of uroporphyrin I was followed by its excretion in the urine (Fischer, 1916). Uroporphyrin III, administered intravenously in the present work, was rapidly excreted in the urine and uroporphyrinogen III was excreted even more rapidly by this route. The difference may be related to solubility. Oxidation of uroporphyrinogen seems to be negligible *in vivo*, since it was excreted in the urine in the reduced form. Conversion of uroporphyrinogen into coproporphyrinogen *in vivo* was not observed. This might be explained by the fact that the excretion of uroporphyrinogen was so rapid that there was not time for such a conversion; another reason might be the low activity of uroporphyrinogen decarboxylase in the liver.

Watson *et al.* (1951) showed that, if perfectly fresh urines are analysed with precautions against oxidation, as much as 66 % of the total porphyrin may be found present in the reduced form. Hoffbauer *et al.* (1953) showed that coproporphyrin, administered intravenously, was excreted exclusively in the bile. Holeček (1957*b*) has reported coproporphyrinogen contents of up to 99 % of the total coproporphyrin in the urine in lead poisoning. Our results show that only coproporphyrinogen passes through the normal renal apparatus and is excreted in the urine, but the recovery of injected doses from the urine was only 6–12 %. Raised faecal excretion of coproporphyrin accounted for another 11 %. Coproporphyrinogen was picked up by the liver and converted enzymically by the mitochondrial system into protoporphyrin, which was excreted in the bile. As shown in Table 4, excretion of coproporphyrinogen rapidly decreased in the bile after 30 min. The rather high amount of coproporphyrin obtained from the calcium hydroxide precipitates may be due to contamination with coproporphyrinogen or, alternatively, to some oxidation occurring during the determination; otherwise, should enzymic oxidation of coproporphyrinogen by the liver be assumed to occur, it would be difficult to explain the continuous formation of protoporphyrin observed over 8 hr. It seems likely, however, that coproporphyrinogen could suffer oxidation in some tissues. The fact that reducing agents enhanced the excretion of coproporphyrinogen supports this idea. Whether or not coproporphyrinogen is oxidized in some tissues, e.g. the liver, is not known. The sum of the amounts of the injected dose which are found in the urine and bile as coproporphyrinogen, coproporphyrin or protoporphyrin only accounts for 20 % of the amount administered (Table 4); the fate of the remainder is unknown. Information on the destruction or deposition of coproporphyrinogen III in various tissues is lacking, but the bone marrow might possibly take up appreciable amounts.

Though this work has established clearly the manner in which various porphyrinogens are dealt with in the body and the conversion of coproporphyrinogen III into protoporphyrin *in vivo*, it has not given any evidence of the metabolic reduction of a porphyrin to its corresponding porphyrinogen. The evidence suggests, in fact, that such conversion does not take place. Oxidation from the porphyrinogen to the porphyrin level, on the contrary, does occur *in vivo*, but the extent to which this oxidation takes place can be influenced, with coproporphyrinogen at least, by such physiological factors as the status of the animal with respect to ascorbic acid or glutathione.

In pathological states, one may find large amounts of coproporphyrin in the faeces without abnormality in the urine, as, for example, in porphyria cutanea tarda hereditaria in remission, and this must probably indicate a disturbance of the cellular oxidation–reduction level such that biosynthesized porphyrinogen becomes oxidized and is allowed to escape from the biosynthetic pathway (Heikel, Lockwood & Rimington, 1958; Rimington, 1961*b*). It is the urinary and faecal excretion of coproporphyrinogen or coproporphyrin which is most informative in this connexion since, as shown in the present work, uroporphyrin and its porphyrinogen are both excreted readily by the kidney whereas neither reduced nor oxidized dicarboxylic porphyrins pass this barrier. Examination of organs *post mortem* for red fluorescence under ultraviolet light may, however, be a valuable guide; when liver tissue of cases of congenital porphyria, acute intermittent porphyria and porphyria cutanea tarda were so examined by Schmid, Schwartz & Watson (1954), it was only the last-named which showed immediate porphyrin fluorescence (Rimington, 1955).

Coproporphyrinuria has for long been recognized as one of the accompaniments of lead poisoning. It seems certain that it is actually the chromogen, coproporphyrinogen III, which is so excreted (Weatherall, 1952; Holeček, 1957*b*). The increase is definitely associated with the elevated coproporphyrinogen III content of the erythropoietic system (Sano, 1958). Should, however, some oxidation to the porphyrin level occur *in vivo*, there will be a diminished urinary excretion of coproporphyrinogen and a rise in the faecal coproporphyrin content, even though an increased synthesis of coproporphyrinogen persists. There may, therefore, be a relation between urinary coproporphyrinogen and the oxidation–reduction level of such systems as GSH–GSSG, NADPH–NADP⁺, NADH–NAD⁺ and ascorbate–dehydroascorbate in lead poisoning.

We have confirmed the observation of Hoffbauer *et al.* (1953) that after carbon tetrachloride poisoning some injected coproporphyrin appears in the urine, and we have established that it is excreted as coproporphyrin and not as coproporphyrinogen—i.e. no reduction has taken place even under these circumstances.

The present work has not brought forward evidence supporting the existence of protoporphyrinogen *in vivo* (Table 4): the exact stage at which oxidation to protoporphyrin occurs is still obscure. Sites in which it occurs in high concentration, e.g. the Harderian gland, show immediate intense fluorescence in ultraviolet light (Rimington, 1955).

Haematoporphyrin is a typical porphyrin on which ready solubility is conferred by its hydroxyl-

containing side chains. We found, nevertheless, that its excretion by the kidney was practically negligible. Kennedy (1954), however, has reported that haematoporphyrin administered intraperitoneally to rats was excreted in large amount in the urine for 6 days. The reason for this difference is not known.

Though answers have been supplied to some of the questions enumerated in our introduction, there still remain important points to be clarified relating to the behaviour of both porphyrins and porphyrinogens *in vivo*.

SUMMARY

1. In normal rabbits, intravenously administered uroporphyrin III and uroporphyrinogen III were both excreted exclusively in the urine.

2. Injected coproporphyrinogen III was excreted in part in the urine, the addition of reducing agents or prior saturation of the animal with ascorbic acid increasing the proportion so excreted.

3. The dicarboxylic porphyrins and porphyrinogens were not excreted in the urine in more than traces after injection.

4. There was a significant urinary excretion of injected coproporphyrin III after carbon tetrachloride poisoning but not after lead poisoning.

5. Injection of coproporphyrin III into the rabbit with a ligated common bile duct was followed by excretion of coproporphyrin III in the urine. The concentration rose during the first 4 hr. after which it remained at a high level. Between 50 and 67% of the dose was so excreted before death. There was coincident bilirubinuria and haematuria.

6. Determination of blood porphyrin or porphyrinogen concentrations after injection of coproporphyrin III or coproporphyrinogen III into normal rabbits and rabbits with surgical ligation of the common bile duct showed that there is an avid uptake of these materials, presumably by the liver, resulting in rapidly falling blood concentrations. When biliary excretion was prevented, however, pigment returned to the blood stream where its concentration remained elevated for a considerable time.

7. The metabolic conversion *in vivo* of coproporphyrinogen III into protoporphyrin has been demonstrated. There was no detectable conversion of uroporphyrinogen III into coproporphyrinogen III.

We thank Professor Nishio, Department of Public Health, Faculty of Medicine, Kyoto University, Kyoto, Japan, for his kindness in allowing one of us (S.S.) to complete in his Department some remaining work. We also thank Dr Nanzyo and Mr Iwata for their technical assistance.

REFERENCES

- Bogorad, L. & Granick, S. (1953). *J. biol. Chem.* **202**, 793.
 Falk, J. E. & Benson, A. (1953). *Biochem. J.* **55**, 101.
 Fischer, H. (1915-1916). *Hoppe-Seyl. Z.* **96**, 148.
 Fischer, H. (1916). *Münch. med. Wschr.* **11**, 377.
 Fischer, H. (1953). *Biochem. Prep.* **3**, 39.
 Fischer, H. & Orth, H. (1943). *Chemie des Pyrrols*, p. 414. Leipzig: Akademische Verlagsgesellschaft; Photo-Litho-print Reproduction, Ann Arbor: Edwards Brother Inc.
 Gray, C. H. & Holt, L. B. (1948). *Biochem. J.* **43**, 191.
 Heikel, T., Knight, B. C., Rimington, C., Ritchie, H. D. & Williams, E. J. (1960). *Proc. Roy. Soc. B*, **153**, 47.
 Heikel, T., Lockwood, W. H. & Rimington, C. (1958). *Nature, Lond.*, **182**, 313.
 Hoffbauer, F. W., Watson, C. J. & Schwartz, S. (1953). *Proc. Soc. exp. Biol., N.Y.*, **83**, 238.
 Holeček, V. (1957a). *Pracov. Lék.* **9**, 513.
 Holeček, V. (1957b). *Brit. J. industr. Med.* **14**, 198.
 Kennedy, G. Y. (1954). Ph.D. Thesis: University of Sheffield.
 Mauzerall, D. & Granick, S. (1958). *J. biol. Chem.* **232**, 1141.
 Nicholas, R. E. H. & Rimington, C. (1952). *Biochem. J.* **50**, 194.
 Rimington, C. (1955). *Endeavour*, **14**, 126.
 Rimington, C. (1961a). *Ass. clin. Path. Broadsheet, N.S.*, no. 36.
 Rimington, C. (1961b). In *Chemical Pathology of the Nervous System*, p. 88. Ed. by Folch-Pi, J. London: Pergamon Press.
 Rimington, C. & Sveinsson, S. L. (1950). *Scand. J. clin. Lab. Invest.* **2**, 209.
 Saillet (1896). *Rev. médecine*, **16**, 542.
 Sano, S. (1958). *Acta haematol. jap.* **21** (suppl.), 337.
 Sano, S. & Granick, S. (1961). *J. biol. Chem.* **236**, 1173.
 Schmid, R., Schwartz, S. & Watson, C. J. (1954). *A.M.A. Arch. intern. Med.* **93**, 167.
 Watson, C. J., de Mello, R. P., Schwartz, S., Hawkinson, V. E. & Bossenmaier, I. (1951). *J. Lab. clin. Med.* **37**, 831.
 Weatherall, M. (1952). *Biochem. J.* **52**, 683.