EXCRETION OF URINARY COPROPORPHYRIN IN LEAD POISONING

PART I: LEVEL OF THE PRECURSOR OF COPROPORPHYRIN AND PREFORMED COPROPORPHYRIN IN FRESH URINE

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In lead poisoning there are, in addition to other changes, disturbances in the metabolism of the pyrrole derivatives of blood pigments, and one of these is a high level of urinary coproporphyrin. The object of this paper was to find in what form coproporphyrin is eliminated in the urine.

Saillet found as early as 1896 that coproporphyrin was partly eliminated in the urine in the form of a non-fluorescent precursor, but this problem has been studied in more detail only in recent years. Watson, de Mello, Schwartz, Hawkinson, and Bossenmaier (1951) found in fresh urine of healthy subjects that the precursor formed more than half of the total value of the coproporphyrin. Weatherall (1952) found in the fresh urine of rabbits, poisoned with lead, as much as 83% of the precursor, with an average value of 49%. In both these papers the extraction method of determination was used, in which, during the extraction, the precursor is transformed to coproporphyrin. Eriksen (1951a) used adsorption on calcium phosphate for the determination of preformed coproporphyrin and found as much as two-thirds of the precursor in the urine of subjects with lead poisoning. He did not work, however, with sufficiently fresh urine. No experimental proof has so far been given of the presence of preformed coproporphyrin in the urine of healthy subjects or in lead poisoning. On the contrary, it was found that injected coproporphyrin was eliminated by the liver (Weatherall, 1952; Goldberg, 1955; Hoffbauer, Watson, and Schwartz, 1953), not by the kidneys. We are of the opinion that the coproporphyrin found in the urine had passed through the kidneys in the form of the precursor. The precursor of coproporphyrin has not been isolated. It appears that it is a four-pyrrole compound which is very readily oxidized to coproporphyrin (Watson *et al.*, 1951), and we have investigated experimentally the

theory that in lead poisoning only the precursor of coproporphyrin and not preformed coproporphyrin is present in fresh urine. In the determination of coproporphyrin in the urine immediate measurement, or the exclusion of light even for very short periods, is imperative.

Experimental

Chemicals and Apparatus.—Chemicals of the maximal available analytical purity were used. Ether was obtained free from peroxidic substances by storage over ferrous sulphate and was always freshly distilled.

The determination of the concentration of coproporphyrin in extracts in hydrochloric acid was performed spectrophotometrically on Beckman's spectrophotometer using the method of Rimington and Sveinsson (1950).

Method.—All determinations were performed in subdued light at a distance of 6 metres from the window. Before chemical treatment each sample of urine was kept in complete darkness. The determination of the quantity of precursor in the alkaline supernatant in Cases 10 *a* to *f* in Table 3 was carried out in a room from which all daylight had been excluded, illuminated by a 25-watt lamp at a distance of 3 metres.

For the determination of preformed coproporphyrin, the quickest available method was used so that the spontaneous conversion of the precursor to coproporphyrin might be minimal. It was also necessary to avoid working in an acid medium where the precursor is readily transformed to coproporphyrin. Adsorption on calcium phosphate in an alkaline medium (Askevold (1951)) was the method used and the calcium phosphate was precipitated within five minutes of micturition.

One drop of 5% hydrogen dipotassium orthophosphate and 1 ml. of 3% calcium chloride were added to 10 ml. of urine. Calcium phosphate was then precipitated by adding 1 ml. of 2 N sodium hydroxide and centrifuging for five minutes. The precipitate was washed with 10 ml. of 0.1 N sodium hydroxide, again centrifuged,

and dissolved in the minimum volume of 2 N hydrochloric acid. Due to the small coproporphyrin content and the great amount of sediment, the spectrophotometric determination could not be carried out directly in this solution. The solution was therefore buffered with sodium acetate to pH 4 to 5 and the coproporphyrin determined after ether-hydrochloric acid extraction. We had established previously that up to 5 µg. of coproporphyrin could be adsorbed quantitatively on to the calcium phosphate precipitate as it was formed in this experiment (Table 1). If the content of the preformed coproporphyrin in the urine is higher than this, the urine must be diluted. In our cases, however, this was not so. It was also necessary to test the behaviour of the precursor during the adsorption of preformed coproporphyrin onto calcium phosphate. The isolated precursor was not available, and an experiment was therefore undertaken to find out whether the precursor was co-precipitated on to calcium phosphate.

TABLE 1
ADSORPTION OF COPROPORPHYRIN ON CALCIUM
PHOSPHATE FROM WATER-MEDIUM AND
FROM URINE

Medium	Coproporphyrin (µg.)		Recovery (%)
	Added	Recovered	
0.1 N HCl + water 1 : 1 (10 ml.)	5-10	4.03	79.0
		4.77	93.5
		3.88	76.1
		4.37	85.7
	0.99	0.97	98.0
		0.96	97.0
		0.98	99.0
		0.96	97.0
	0.102	0.098	96.1
		0.094	92.2
		0.102	100.0
		0.094	92.2
0.1 N HCl + urine 1 : 1 (10 ml.)	5-10	4.81	94.3
		5.04	98.8
		4.90	96.1
		4.80	94.1
	0.99	1.01	102.0
		0.99	100.0
		1.02	103.0
		0.97	98.0
	0.102	0.098	96.1
		0.110	107.8
		0.118	115.7
		0.086	84.5

(1) Freshly passed undiluted urine, and the same urine diluted 1 : 1 and 1 : 3 with water, were treated with the calcium phosphate precipitation method described above.

(2) Freshly passed undiluted urine was treated with the amounts of 3% calcium chloride and 5% hydrogen dipotassium orthophosphate described above (basic), also with twice and four times these amounts (Table 2).

Table 2 shows that the precursor is partly co-precipitated on to the calcium phosphate, the amount co-precipitated being independent of the dilution of the urine, but increasing with the amount of calcium phosphate precipitated. It is not possible to determine the range of the co-precipitation as the precursor is very

TABLE 2
CO-PRECIPITATION OF THE PRECURSOR OF
COPROPORPHYRIN ONTO CALCIUM PHOSPHATE

Dilution of Urine	Calcium Phosphate Content	Sample	
		1	2
		Total Coproporphyrin in $\mu\text{g./ml.}$ of Urine	
		1.450	3.760
		Preformed Coproporphyrin Calculated as ($\mu\text{g./ml.}$) of Undiluted Urine	Coproporphyrin Calculated as ($\mu\text{g./ml.}$) of Undiluted Urine
—	Basic	0.077	0.174
1 + 1	"	0.069	0.213
1 + 3	"	0.070	0.169
—	Twofold	0.102	0.236
—	Fourfold	0.131	0.298

rapidly transformed into coproporphyrin and the results are thereby raised. The smallest possible quantity of reagents was therefore chosen, and the calcium phosphate was precipitated immediately after adding hydroxide. In this way all the preformed coproporphyrin was determined quantitatively, but the value found was increased by part of the co-precipitated precursor, which had been rapidly transformed to coproporphyrin. In our view this is the most suitable method for the determination of preformed coproporphyrin in the urine, since we found lower values of preformed coproporphyrin in the urine than have been so far reported in the literature (Watson *et al.*, 1951; Weatherall, 1952; Eriksen, 1951a). For the determination of total coproporphyrin, *i.e.*, the sum of preformed coproporphyrin and the precursor, the ethylacetate-hydrochloric acid method of extraction was used. For the conversion of all the precursor to coproporphyrin, oxidation with iodine (Watson *et al.*, 1951) was used. The precursor content was calculated by subtraction of the amount of preformed coproporphyrin determined by adsorption on calcium phosphate from the amount of total coproporphyrin.

Experiments were carried out to determine the quantity of the precursor not adsorbed on calcium phosphate but left in the alkaline supernatant. This was done by acidifying the supernatant with acetic acid and extraction with ethylacetate and hydrochloric acid. For the conversion of all the precursor to coproporphyrin oxidation with iodine was used.

All determinations of preformed and total coproporphyrin were carried out twice in parallel. The results differed by a few per cent; at low values of preformed coproporphyrin only the difference increased to more than 10%, when the extraction of coproporphyrin was as low as a few hundredths of a microgram. In working with normal urines, 20 ml. of urine was always used.

Results and Discussion

In Table 3 are given the amounts of total and preformed coproporphyrin found in the fresh urine of 28 samples. The amount (Col. v) of the precursor is calculated from the difference of both values and also expressed as a percentage of total coproporphyrin. Column (vi) of Table 3 shows the amount

TABLE 3
EXCRETION OF PRECURSOR AND PREFORMED COPROPORPHYRIN IN URINE

Sample (i)	Total Coproporphyrin ($\mu\text{g./ml.}$) (ii)	Preformed Coproporphyrin		Precursor Calculated (%) (ii)-(iii) (v)	Precursor Found ($\mu\text{g./ml.}$) (vi)	Preformed Coproporphyrin + Precursor Found		Remarks (ix)
		$\mu\text{g./ml.}$ (iii)	% Total Coproporphyrin (iv)			$\mu\text{g./ml.}$ (vii)	% of Total Coproporphyrin (viii)	
1	0.203	0.020	9.9	90.1	—	—	—	Lead poisoning
2	0.232	0.022	9.5	90.5	—	—	—	
3	0.326	0.003	0.9	99.1	—	—	—	
4	0.352	0.012	3.4	96.6	0.320	0.332	94.3	
5	1.410	0.077	5.5	94.5	1.262	1.339	95.0	
6	1.450	0.102	7.0	93.0	—	—	—	
7	2.700	0.041	1.5	98.5	1.400	1.441	53.4	
8	3.730	0.448	12.0	88.0	—	—	—	
9	3.760	0.174	4.6	95.4	2.070	2.244	59.7	
10a	1.105	0.030	2.7	97.3	0.841	0.871	78.8	
10b	1.010	0.030	3.0	97.0	0.879	0.909	90.0	
10c	0.975	0.032	3.3	96.7	0.795	0.827	84.8	
10d	0.927	0.026	2.8	97.2	0.784	0.810	87.4	
10e	0.966	0.031	3.2	96.8	—	—	—	After treat- ment
10f	1.020	0.026	2.6	97.4	0.742	0.768	75.3	
11	0.205	0.006	2.9	97.1	—	—	—	
12	0.125	0.019	15.2	84.8	—	—	—	
13	0.124	0.013	10.5	89.5	—	—	—	Normal urines
14	0.052	negat.	—	100	—	—	—	
15	0.132	0.018	13.6	86.4	—	—	—	
16	0.095	0.019	20.0	80.0	—	—	—	
17	0.092	0.017	18.5	81.5	0.043	0.060	65.2	
18	0.091	0.002	2.2	97.8	—	—	—	
19	0.086	0.020	23.3	76.7	0.039	0.059	68.6	
20	0.063	0.016	25.4	74.6	—	—	—	
21	0.050	0.011	23.0	77.0	—	—	—	
22	0.042	0.007	16.6	83.4	—	—	—	
23	0.029	0.006	20.7	79.3	—	—	—	

of precursor as determined by direct analysis in alkaline supernatant. Samples No. 10 a to f represent analyses of samples of urine which were taken at 10-minute intervals from one patient by means of a rubber catheter in a nitrogen atmosphere in subdued light.

Table 3 shows that both in lead poisoning and in normal urines a large proportion of coproporphyrin is excreted as precursor, and the high coproporphyrin level in the urine in lead poisoning is thus due to the excreted coproporphyrin precursor. The percentage of preformed coproporphyrin which we have found is much lower than that found by earlier authors (Watson *et al.*, 1951; Weatherall, 1952; Eriksen, 1951a). In our view these lower results were obtained by improved analytical techniques and by treating the urine immediately after micturition. It is certain that the actual amount of preformed coproporphyrin in urine is still lower but it cannot at present be shown to be zero. The relatively high percentages of preformed coproporphyrin found even in freshly passed normal urine (Table 3) give no indication whether these amounts, which are very small, originated from the precursor in the bladder or whether they were excreted by the kidney.

Table 3 also shows that in the determination of coproporphyrin-precursor it is partially broken down in alkaline supernatant and therefore it is not possible to determine the precursor quantitatively

by direct analysis. These results do not entirely agree with those of Eriksen (1951b, 1952).

Determinations of total coproporphyrin in fresh urine were made, as well as of the precursor in alkaline supernatant, both with and without using iodine as oxidant. In contrast with normal urines, where the difference of the analyses with and without oxidation of iodine was about 30%, this difference in the urines of cases of lead poisoning was only 2 to 10%.

The breakdown of the precursor and its conversion into coproporphyrin in untreated urine were further studied. The urine in test tubes was (i) exposed to diffuse daylight directly in a window, (ii) kept in the dark at room temperature, (iii) kept in the dark in the refrigerator. The preformed total coproporphyrin levels were determined after two, six, and 24 hours. The results are given in Table 4.

The effect of daylight appeared to be to cause a rapid disintegration of the precursor. (In an ether or ethylacetate solution, light causes a rapid conversion of the precursor to coproporphyrin.) In one experiment the total coproporphyrin level fell to zero in the course of two hours. In the dark, at room temperature, the precursor was for the most part converted to coproporphyrin and disintegrated only partially. In the refrigerator, nearly all the precursor changed to coproporphyrin and the losses were very small. These results are a very important

TABLE 4
BREAKDOWN AND CONVERSION OF THE PRECURSOR TO COPROPORPHYRIN

Time (hours)	Preformed Coproporphyrin (μg./ml. Urine)			Total Coproporphyrin (μg./ml. Urine)		
	Conditions in Ageing Urine					
	In the dark in refrigerator	At room temperature		In the dark in refrigerator	At room temperature	
		In the dark	In the test tube in a window		In the dark	In the test tube in a window
0	0.077			1.37		
2	0.145	0.168	0.120	1.41	1.38	0.31
6	0.532	0.490	0.060	1.38	1.21	0.12
24	0.920	0.742	—	1.39	1.08	—

finding for those laboratories in which routine determinations of coproporphyrin in the urine are undertaken. In all determinations of coproporphyrin it is imperative to keep the urine away from light immediately after micturition.

Summary

The total coproporphyrin in completely fresh urine in lead poisoning was found to be formed of 1 to 12% preformed coproporphyrin, and 88 to 99% precursor of coproporphyrin. Preformed coproporphyrin was not excreted in increased amount in lead poisoning. The increased level of total urinary coproporphyrin in lead poisoning is due to excessive excretion of the coproporphyrin precursor.

The precursor is, after micturition, rapidly transformed to coproporphyrin in the dark in the presence of oxygen in the air. In the light it disintegrates rapidly to non-porphyrin substances. Urines were examined in which this disintegration

took place almost completely when they were exposed to the effect of diffuse daylight for two hours. In these cases the content of total coproporphyrin fell to only a small percentage of the original value. It is imperative that urine samples for coproporphyrin estimations are not exposed to light even for a short period.

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PART II: DISTRIBUTION OF URINARY COPROPORPHYRIN ISOMERS I AND III

In our previous paper we demonstrated that in lead poisoning the high coproporphyrin level is due to excessive excretion of the urinary coproporphyrin precursor. In our present work we studied the problem of the transformation of the precursor to coproporphyrin isomers I and III (Fig. 1a and 1b).

The view has been generally accepted that the coproporphyrin in the urine in lead poisoning is the isomer III. This would mean that the coproporphyrin precursor (coproporphyrinogen) is converted to the isomer III when analysing the urine. Kench, Lane, and Varley (1952) found, however, an average of 30% coproporphyrin I in lead poisoning. Since the chemical composition of the precursor (coproporphyrinogen) is not known, the possibility must be considered of the different ratio of the two isomers arising as a result of the way in which the urine is treated.

It is probable that coproporphyrinogen in normal and pathological urine is a four-pyrrolic compound (Watson, de Mello, Schwartz, Hawkinson, and Bossenmaier, 1951) (Fig. 1c), which could only give rise to a coproporphyrin isomer corresponding to the original isomer of the coproporphyrinogen regardless of the way in which the urine was treated. We must also consider the possibility of the existence of other precursors of coproporphyrins which could form coproporphyrins of different isomeric configuration according to the chemical treatment. Thus, for example, on condensation of the monopyrrolic porphobilinogen, isolated from the urine in acute porphyria, uroporphyrin I is mostly formed in an alkaline medium and in an acid medium uroporphyrin III (Cookson and Rimington, 1954) (Fig. 1d to f).

The same argument for the distribution of

FIG. 1 a to c.—Chemical groups:

M = methyl P = propionic acid residue

(a) Coproporphyrin I

(b) Coproporphyrin III

(c) Assumed precursor of Coproporphyrin III

FIG. 1 d to f.—Chemical groups:

O = residue of acetic acid

P = residue of propionic acid

(d) Porphobilinogen

(e) Uroporphyrin I

(f) Uroporphyrin III

coproporphyrin isomers I and III is also valid for normal urines, where to date nothing is definitely known on the proportion of the two isomers, although this question has been studied by several authors. In a recent paper, Comfort, Moore, and Weatherall (1954) reported a great predominance of isomer III over isomer I, but in view of the conflicting findings of other authors they admit both the possibility of different ratios of the two isomers caused by conditions during the conversion of the precursor, and such a great individual variability in the mutual ratio of the two isomers that it cannot be said whether one of the isomers usually predominates.

We attempted in our present work to decide experimentally whether the relative representation of the two coproporphyrin isomers is produced by the nature of the precursor itself, or whether it depends on the treatment of the urine when isolating the coproporphyrin.

Experimental Procedure

Chemicals.—Chemicals of the maximal available analytical purity were used. Coproporphyrin I was isolated from calf meconium; coproporphyrin III was obtained from a filtrate of diphtheria bacteria, uroporphyrin from the urine of a patient with porphyria. The composition of isomers of the lutidine used was not known. After drying with solid potassium hydroxide, the fraction used had a practically constant boiling point (153.5 to 153.6° C. corr.) at a pressure of 740 mm. Hg and was unlimitedly miscible with water at temperatures lower than + 15° C.

Method.—Fresh urine was divided into five parts and each part was treated immediately after micturition so that all the routine methods of extraction of urinary coproporphyrin were used. Thus the conversion of the precursor was brought about under the following conditions:—

(1) Spontaneous conversion in the untreated urine (the most frequent way of conversion in practice); (2) conversion in urine made alkaline by the addition of sodium carbonate (conservation of urine according to Schwartz, Zieve, and Watson, 1951); (3) conversion in untreated urine by heating to 100° C. (method used for the conversion of porphobilinogen into uroporphyrin (Grieg, Askevold, and Sveinsson (1950)); (4) oxidation with iodine in ethylacetate (oxidation of the precursor according to Watson *et al.*, 1951); (5) conversion by the action of hydrochloric acid on ethylacetate extract (routine method of extraction of coproporphyrin from ethylacetate into hydrochloric acid).

In this way, large volumes of urine of three cases of lead poisoning were treated and the amounts of porphyrin (mg.) obtained were purified by column chromatography and in the obtained coproporphyrin fraction both isomers were estimated by paper chromatography. Then crystalline methylesters of coproporphyrins were prepared in which the form of the crystals could be assessed and the melting point determined.

Later, small volumes of urine were analysed and both isomers determined only by paper chromatography. In these cases the methods of isolation were so chosen that the conversion of the precursor took place. (6) after the action of 0.1 to 0.2 N sodium hydroxide on urine with subsequent acidification (Eriksen, 1952); (7) in urine acidified by hydrochloric acid to a concentration of 0.1 to 0.2 N (this was used as a complementary method); (8) in ethylacetate by oxidation with iodine and by the action of 2 N hydrochloric acid (Watson *et al.*, 1951).

The chromatographic separation of coproporphyrin isomers I and III on paper was carried out according to the method of Chu, Green, and Chu Ju-Hwa (1951) with pure preparations isolated from large volumes of urine and with all techniques according to our modification of the method of Nicholas and Rimington (1949). (The method is very similar to that of Falk, which appeared during our investigations (Falk, 1955; Falk, Dresel, Benson, and Knight, 1956); the principle is described later.) The separation of non-purified coproporphyrins I and III extracted from small volumes was carried out only according to our method, as we demonstrated that in the presence of sediment from the urine the results by the method of Chu *et al.* are not reproducible, whereas the separation of free porphyrins by the modified method of Nicholas and Rimington is, even under these conditions, reproducible.

The identification of individual porphyrins was carried out by parallel chromatography of a portion of the sample with the addition of standard porphyrin, since the R_F is changed with a small alteration in the composition of the mixture, temperature, concentration of ammonia, and the presence of foreign substances in the sample. For these reasons, and because the behaviour of isomers of other porphyrins was not tested, the number of carboxyl groups cannot be reliably ascribed, on the basis of the R_F values, to porphyrins in the unknown spot.

The chromatographic separation of free coproporphyrin isomers I and III in the system lutidine-water was undertaken as follows:—

Solutions of porphyrins in 2 N ammonia were placed on Whatman paper No. 1 44 cm. long (*i.e.*, the width of the sheet), on a line at a distance of 3 cm. from the edge. The minimal distance of the spots should be 18 mm., when the placed quantities of porphyrins are about 0.8 μ g. The areas of the spots are of no importance.

The development was performed by ascending technique in a completely darkened developing chamber. The solvent used was a lutidine-water mixture (35+25 volume parts). It is not necessary to equilibrate the developing chamber or the paper before the development. Immediately after the solvent was poured into the solvent trough, and the paper irrigated with the solvent, a dish with several millilitres of 25% ammonia was placed in the developing chamber, the chamber was closed and the chromatogram left for 45 hours or longer to develop. When the run was completed the chromatogram was taken out, dried in the air, and the spots marked in pencil in ultra-violet light. The R_F values of the examined porphyrins are given in Table 1.

In order to obtain accurate identification of individual

TABLE 1
R_F OF FREE PORPHYRINS IN THE LUTIDINE-WATER (35+25) ASCENDING TECHNIQUE

R _F	Uroporphyrin	Coproporphyrin I	Coproporphyrin III	Protoporphyrin IX	Unidentified Impurities from Used Coproporphyrin I according to Decreasing Intensity of Spots			
					1	2	3	4
	0.02	0.42	0.48	0.81	0.37	0.56	0.32	0.26

porphyrins, and to exclude the interference of undefined porphyrins, a part of the examined material with a standard porphyrin as control was run simultaneously through the developing chamber.

In order to detect very small quantities of a certain porphyrin in the presence of large quantities of other porphyrins, about 3 to 5 µg. of the porphyrins mixed are placed at a distance of 3 to 4 cm. from each other. In this case, the developed spots of coproporphyrin isomers I and III are not completely separated.

The best separation of coproporphyrin isomers I and III was achieved when the chromatogram ran at 21° C. ± 0.5. Less than 5% of the isomer I and III was detectable in a mixture, when this temperature was maintained.

Treatment of Large Urine Volumes.—In these instances, the preformed coproporphyrin was not removed.

(1) *Conversion of Precursor in Untreated Urine.*—The first portion of urine was kept in a refrigerator for several days, then acidified with acetic acid when 20 g. talc was added to 1 litre of urine. (Talc is commonly used for the adsorption of porphyrins. When examining the conditions of adsorption, we found that for the quantitative adsorption of porphyrins from the medium buffered with an acetate buffer, at least 2,000 times larger amounts of talc than the porphyrin content, and two to three hours of intensive shaking were needed. From a medium of 0.1 N hydrochloric acid, 10,000 times more talc was needed, but shaking for two minutes was sufficient. At least 10 g. of talc for 1 litre of urine was used.) After shaking for three hours, the talc with the adsorbed porphyrin was filtered off and dried. The adsorbed porphyrin was converted to methylester (Grinstein, Schwartz, and Watson, 1945) chromatographically purified on aluminium oxide (Nicholas, 1951) and recrystallized. On chromatographic purification on aluminium oxide, only the coproporphyrin fraction was trapped. The fractions with other porphyrins were not further analysed.

(2) *Conversion of Precursor in Alkalized Urine.*—In the second portion of urine, sodium carbonate was dissolved to a concentration of 1 to 5% immediately after micturition, and the further procedure was the same as in (1) above.

(3) *Conversion of Precursor in Untreated Urine by Heating.*—The third portion of urine was heated for 20 minutes on a boiling-water bath immediately after micturition, and then immediately treated in the same way.

(4) and (5) *Conversion of Precursor in Ethylacetate by Oxidation with Iodine and Action of Hydrochloric Acid.*—

In the fourth and fifth portions of the urine, the coproporphyrin was isolated by extraction with ethylacetate-hydrochloric acid. In the fourth portion, oxidation with iodine according to the method of Watson *et al.* (1951) was carried out. The extracts in hydrochloric acid were buffered with sodium acetate, coproporphyrin was adsorbed on to talc and further treated as in (1) above.

Using methods 1 and 2, 10 litres of urine from a patient (A) with lead poisoning were treated and 1 litre of urine from two further patients with lead poisoning (B, C) and 10 litres of mixed urine from six healthy subjects. Using methods 3, 4, and 5, 1 litre of urine each from patients A, B, and C were treated.

Treatment of Small Urine Volumes.—In these instances the preformed coproporphyrin was removed.

(6) *Removal of Preformed Coproporphyrin and Conversion of Precursor after Action of 0.1 to 0.2 N Sodium Hydroxide.*—(a) from quite fresh urine (5 min. after micturition at the latest) when the preformed coproporphyrin was adsorbed onto calcium phosphate according to the method of Askevold (1951), as described in our first communication. After centrifugation, the alkaline liquid (sodium hydroxide content 0.1 to 0.2 N) was decanted above the adsorbate, acidified with acetic acid and the coproporphyrin determined by extraction in ethylacetate-hydrochloric acid. (b) The adsorbate on calcium phosphate was dissolved in 2 N hydrochloric acid, buffered with sodium acetate to pH 4 to 5 and treated by the same extraction method. The final extract in hydrochloric acid was evaporated to dryness in nitrogen at 80° C., dissolved in one drop of 2 N ammonia and chromatographed.

(7) *Conversion of Precursor in Acidified Urine.*—The second portion of the urine was acidified with hydrochloric acid to a concentration of 0.1 to 0.2 N. After 24 hours in the refrigerator, it was buffered with sodium acetate and extracted with ethylacetate-hydrochloric acid. The further treatment was the same as in 6(b) above.

(8) *Conversion of Precursor in Ethylacetate by Oxidation with Iodine and by Action of Hydrochloric Acid.*—The third portion of the urine was treated by extraction as described in (4) above.

Using the procedure 6 to 8, 10 ml. samples of completely fresh urine of patients with symptoms of lead poisoning and 20 ml. samples of completely fresh urine of healthy subjects were treated. Using methods 6 and 8, nine samples each of normal urine and of urine of subjects with symptoms of lead poisoning were treated. Using method 7, three samples of normal urine and of urine of subjects with symptoms of lead poisoning were treated. In the procedure 7 and 8, the preformed coproporphyrin had not been previously removed.

Results and Discussion

Methylesters of coproporphyrins, isolated from the urine in lead poisoning, were all readily soluble in methyl alcohol, so that it was not possible to obtain even small amounts of methylester of isomer I, which is relatively insoluble in methyl alcohol. During crystallization, no typical needle-like crystals of methylester of the isomer I were found (Fig. 2, 3, and 4) so that the presence of an important amount of isomer I could not be demonstrated.

The melting point of the preparations studied indicates throughout the presence of isomer III (Table 2).

In paper chromatography of coproporphyrins Chu *et al.* found no coproporphyrin I. According to our experiments, no amount of a coproporphyrin isomer smaller than 20% could be found by this method in a mixture of the two isomers. Using our chromatographic method in lead poisoning, however, the presence of small amounts of coproporphyrin I was found in all instances. This finding is not in disagreement either with the values of melting points found, or with the observed form of crystals, as the chromatographic analysis is more sensitive for the detection of small amounts of coproporphyrin I in a mixture with an excess of coproporphyrin III (Jope, and O'Brien, 1945).

FIG. 2.—Tetramethylester of coproporphyrin I $\times 180$.

FIG. 3.—Tetramethylester of coproporphyrin III $\times 180$.

FIG. 4.—Tetramethylester of coproporphyrin isolated from the urine in lead poisoning $\times 180$.

FIG. 5. Paper chromatography of porphyrins in the system lutidine-water.

1. Standard mixture of coproporphyrin I and III.
2. Porphyrins isolated from normal human urine.
3. Porphyrins from the urine in lead poisoning (performed coproporphyrin and porphyrins produced in ethylacetate by action of iodine and hydrochloric acid).
4. Porphyrins produced from precursor in the urine in lead poisoning by the action of sodium hydroxide and acetic acid.
5. The same specimen as at (4) with addition of standard coproporphyrin III.
6. The same specimen as at (4) with addition of standard coproporphyrin I.

FIG. 6.—The relation of R_F of the spots of free porphyrins in the system lutidine-water to the number of carboxyls.

On the chromatograms, in all instances two faint spots were also visible, corresponding to porphyrins with five to six carboxyl groups (Fig. 5, 6).

In some cases, where the precursor was converted to coproporphyrin after the action of alkali, very intensive spots appeared on the chromatogram, corresponding to porphyrins with two to three carboxyl groups (Fig. 5, 6).

It may be concluded that the coproporphyrin precursor excreted in lead poisoning is mainly the precursor of coproporphyrin III. In chemical treatment of the urine, its partial decarboxylation and the production of porphyrins with a smaller number of carboxyls may occur, but the ratio of coproporphyrin I and III remains unchanged.

Methylesters of coproporphyrins, isolated from normal urine after acid and alkali treatment, were

poorly soluble in methyl alcohol. During crystallization, needle-like crystals of the type of the methylester of coproporphyrin I (Fig. 7) were obtained.

The melting-points of methylesters of coproporphyrins, isolated from normal urine, lay between the values of methylesters of isomers I and III (Table 2), and were evidence of the presence of a large amount of isomer I.

The chromatographic analysis of porphyrins isolated from normal urine showed that the relation

FIG. 7.—Tetramethylester of coproporphyrin isolated from normal urine $\times 180$.

TABLE 2

MELTING POINTS ($^{\circ}\text{C}$.) OF COPROPORPHYRIN METHYLESTERS, ISOLATED FROM THE URINE OF PATIENTS WITH LEAD POISONING AND FROM NORMAL URINE

Conversion of the Precursor	Specimen							
	1		2		3		Normal Urine	
	Crystallization	Re-melting	Crystallization	Re-melting	Crystallization	Re-melting	Crystallization	Re-melting
Untreated urine in refrigerator	154	158	138–144	143–145	149–156	160–162	223–226	227
Urine alkalinized with sodium carbonate in refrigerator	172–178	165–195	156–158	159–162	157–160	160–164	225–229	224–226
Fresh urine heated to 100°C . for 20 min.	145–150	165	136–139	149	147	159	—	—
Action of iodine and hydrochloric acid on the ethylacetate extract	161–174	174–187	138	149–155	152–153	162–166	—	—
Action of hydrochloric acid on ethylacetate extract	159–171	179	132–134	149	151–152	158	—	—
The range usually given:								
Coproporphyrin III	135–153	158–178	—	—	—	—	—	—
Coproporphyrin I	251–254	—	—	—	—	—	—	—
Our preparations:								
Coproporphyrin III	150	178						
Coproporphyrin I	249	—						

All melting-points are corrected.

of isomers of coproporphyrin I and III in all instances equalled about one (Fig. 5). Further, in all cases, two faint spots were observed, corresponding to porphyrins with 5 to 6 carboxyl groups, as in the urine in lead poisoning.

In paper chromatography of the preformed porphyrin, isolated by adsorption onto calcium phosphate, only coproporphyrin III was found in the urines treated in lead poisoning. Isomer I was probably present, but its presence could not be confirmed owing to the interference of sediment. When treating normal urine, equal amounts of preformed coproporphyrin I and III were found.

Summary

The conversion of the coproporphyrin precursor in the urine both in lead poisoning and in healthy subjects was studied.

It was found that coproporphyrin precursor in the urine in lead poisoning was for the most part the precursor of coproporphyrin III and to a very small extent precursor of coproporphyrin I. The type of chemical treatment of the urine had no effect on the relative values of the resulting isomers I and III. In an alkaline medium, partial decarboxylation and the production of other porphyrins took place with the conversion of the precursor, but the relative values of the coproporphyrin isomers were not changed.

The coproporphyrin precursor in the urine of healthy subjects was, on the other hand, formed by both isomers in equal amounts. The type of chemical treatment of the urine was here also of no effect on the ratio of the resulting isomers.

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