CONCERNING THE NATURALLY OCCURRING PORPHYRINS: *I. The Isolation of Coproporphyrin I from the Urine in a Case of Cincophen Cirrhosis*

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CONCERNING THE NATURALLY OCCURRING PORPHYRINS

I. The Isolation of Coproporphyrin I from the Urine in a Case of Cincophen Cirrhosis [1]

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Evidence for a dualism of the porphyrins in nature has been repeatedly presented by Hans Fischer (1, 2, 3). This means that both plant and animal organisms are capable of the formation of porphyrins of two isomeric series, those corresponding to aetioporphyrin III, including hemoglobin and chlorophyll, and those of the aetioporphyrin I type, the function of whose representatives is as yet very little understood. The aetioporphyrins, of which there are four, have only methyl and ethyl groups in varying position on the porphyrin ring. Because of this relative simplicity Fischer has classified the isomers of the other porphyrins according to which aetioporphyrin their structure corresponds. The aetioporphyrins are artificial. Porphyrins corresponding in structure to aetioporphyrins I and III have been found in nature, those corresponding to II and IV have only been obtained artificially. Fischer has pointed out that it is chemically inconceivable for porphyrins of the one type to be transformed into those of the other except by complete destruction and re-synthesis. Thus it is necessary to look upon the formation of the two series occurring in nature as independent processes. Porphyrins corresponding to aetioporphyrin I were first isolated from the urine and feces of a case of congenital porphyrinuria by H. Fischer (4). These were named coproporphyrin I and uroporphyrin I, the former having four methyl and four propionic acid groups, the latter eight carboxyl groups. Several instances of the same disease have since been studied by Fischer and Duesberg (5), and also Van den Bergh (6), in which identical porphyrins were excreted. However, one instance was reported by each in which coproporphyrin III was isolated. Thus evidence for the existence of two chemically different, but clinically indistinguishable types of idiopathic porphyrinuria was presented. In only one other condition has coproporphyrin III been isolated: namely, by Grotepass (7) from the urine of a patient with lead poisoning. This finding was substantiated by Fischer and Duesberg (5) in a study of experimental lead poisoning in rabbits. Traces of coproporphyrin occur in normal urine, as recognized by Schumm (8), also by H. Fischer and Zerweck (9), but whether of the aetioporphyrin I or III type has not been determined. As Kämmerer (10) has recently observed, this remains one of the chief difficulties in the porphyrin problem, and the extremely small amounts in the normal urine make this obstacle almost insurmountable. H. Fischer (4) estimated that at least 1000 liters of normal urine would have to be employed in order to isolate sufficient porphyrin for microanalysis. The increase of porphyrin in the urine in jaundice and liver disease of various types has long been recognized (Garrod (11), Günther (12)). Until now porphyrins have not been isolated from such instances. In the present investigation a porphyrin has been isolated from the urine of a patient suffering from cirrhosis of the liver, probably caused by cincophen. This porphyrin has been identified by virtue of ester melting point and spectroscopic characteristics as coproporphyrin I.

MATERIAL AND METHODS

A description of the clinical and anatomical findings in the patient from whose urine coproporphyrin I was isolated, follows:

White male, aged 52. The patient came to the hospital because of jaundice and anorexia of eight weeks duration. For two years he had been troubled by rheumatism. To alleviate this he had taken a patent medicine for three months prior to the onset of jaundice. Chemical examination of this medicine indicated the presence of considerable amounts of cincophen. The jaundice

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was painless. The stools were clay colored and the urine dark brown in color. There was marked ascites which recurred rapidly following each abdominal paracentesis. There was increasing edema of the legs. Hemorrhoids were noted. A definite hemorrhagic tendency was observed, characterized by petechiae and epistaxis. Neither liver nor spleen was palpable. The patient was apathetic, and during the last few days of life was difficult to arouse. Death occurred after a period of coma of about 36 hours duration. *Laboratory examinations:* The hemoglobin was 88 per cent (Sahli). The red blood cells numbered 4,500,000. Another hemoglobin determination one week before death was 96 per cent (Sahli). The leukocytes were 13,500, on another occasion 12,500; the neutrophiles 84 per cent and the lymphocytes 16 per cent. The total leukocyte count one week before death had decreased to 7,800. The blood platelets were 200,000 per cu. mm. The coagulation time of blood obtained by ear lobe puncture was 1½ minutes; the bleeding time was 5 minutes. The capillary resistance test was strongly positive. The icterus index shortly after admission to the hospital was 140 units. The Van den Bergh reaction was of the biphasic prompt, direct type. Five days before death the icterus index was 110 units. The blood Wassermann and Kahn reactions were negative. The urine was acid in reaction, the specific gravity varying from 1.017 to 1.023. The Gmelin test for bilirubin was strongly positive. The urobilinogen in the urine was increased in amount, on one occasion being 4.3 mgm. per day, a week later 27.4 mgm. per day. The method used was a modification of one previously described by the writer (13). The upper limit of normal with this method is believed to be 1.5 mgm. per day. Over a four day period at about the time of the second urine urobilinogen estimation, the average daily excretion of urobilinogen in the feces was found to be 33 mgm. The normal range is from 100 to 250 mgm. per day, using the method just mentioned. These findings indicated an incomplete obstruction to the outflow of bile, and considerable diffuse liver injury. The stools did not contain demonstrable occult blood by the benzidine test.

Necropsy revealed advanced cirrhosis of the liver. The liver weighed 1125 grams, was quite firm and exhibited diffuse fine lobulation. The spleen weighed 300 grams. Microscopically a marked portal cirrhosis was seen in the liver, characterized by very marked fibrosis, moderate lymphocytic infiltration, and extensive bile duct proliferation in the portal spaces. Many of the bile ducts were dilated and filled with bile, as though definite obstruction existed distal to them. The liver parenchyma of the central portion of the lobules appeared to be quite normal. No necrosis was seen and fatty metamorphosis was relatively small in amount. Nothing from an anatomical standpoint served to distinguish this from an ordinary advanced portal cirrhosis.

The method used for the isolation of the coproporphyrin was a modification of that employed by H. Fischer and Duesberg (5). Each 24-hour urine sample for an eight day period was strongly acidified with glacial acetic acid in a 5 liter separatory funnel, and shaken with ⅓ to ½ its volume of ether. (Care was observed at first during this shaking, because of the large amounts of CO_2 liberated on acidification.) Emulsions were broken by addition of small amounts of alcohol. It was occasionally necessary to resort to filtration after shaking with talc. The ether was repeatedly washed with distilled H_2O and was next extracted a number of times with small amounts of 2 per cent HCl. This 2 per cent HCl from each 24-hour urine was united, and extracted repeatedly with chloroform, which removed urobilin and mesobiliviolin (14) as well as most of a small amount of chloroform soluble porphyrin which was present. It was then diluted ten times (to 0.2 per cent HCl) and again extracted with chloroform. This completed the removal of the chloroform soluble porphyrin. This porphyrin was probably protoporphyrin since on later fractionation it was found to leave 2 per cent HCl for chloroform. Nevertheless, its absorption spectrum did not correspond entirely with that of protoporphyrin, but the amount was much too small to permit further purification, so that its identity remained doubtful. The remaining aqueous solution was made negative to congo paper by addition of an excess of solid sodium acetate. Twenty to 25 cc. more of glacial acetic acid were also added. Repeated ether extraction was then carried out. The ether was united, washed with water repeatedly, and the coproporphyrin extracted with 2 per cent HCl. After nearly neutralizing with 10 per cent NaOH, it was again taken into ether. Next it was quantitatively removed from the ether with 10 per cent NaOH. This was allowed to stand over night, but the sodium salt was entirely soluble, as H. Fischer and Kirrmann (15) have shown to be true of coproporphyrin, but not of the porphyrins more closely related to hemin, such as protoporphyrin, or deuteroporphyrin. Upon making this NaOH solution barely acid with 10 per cent HCl, the coproporphyrin was again taken into ether, and the 2 per cent HCl-ether fractionation was once more repeated. The final ether solution was dried over anhydrous sodium sulphate, filtered, and concentrated to a very small volume on the water bath. The free coproporphyrin crystallized in fine needles. This material was esterified by standing over night in methyl alcohol, which had just previously been saturated in the cold with dry HCl gas. The ester was purified, and crystallized out of chloroform-methyl alcohol, according to H. Fischer's method (4, 16). After repeated recrystallization, 1.2 mgm. of material were obtained. In handling such small amounts the use of a microtechnic is obligatory. This is particularly true with reference to filtration; funnels of 1.5 to 2 cm. in diameter with very small headed glass tacks and round filter papers 4 to 5 mm. in diameter are employed with suction. A test tube with a side arm serves to receive the mother liquor. In this way a fraction of a milligram may readily be recrystallized several times. On standing, the separation of crystals out of chloroform-methyl alcohol is almost quantitative. After saponification of a small amount of the ester thus

obtained by allowing it to stand dissolved in 25 per cent HCl over night, sufficient sodium acetate was added to make the solution negative to congo paper, and the free porphyrin was taken into ether. This acetic and ether solution served for a spectrometric study of the porphyrin. For this purpose a Zeiss grating comparison spectrometer was employed.

In the above described separation of the porphyrins, a more complete fractionation was effected by continuing the extractions until the absence or extreme faintness of the red fluorescence in ultra-violet light, of the porphyrin being extracted, indicated that the extraction was complete. This red fluorescence persists long after the porphyrin color and absorption spectrum has ceased to be visible. As a source of ultra-violet light, a small Leitz hand arc lamp was used, a copper sulfate solution being employed to filter out a considerable part of the light of longer wave length.

RESULTS

The crystals of the porphyrin ester obtained have the appearance shown in Figure 1. The

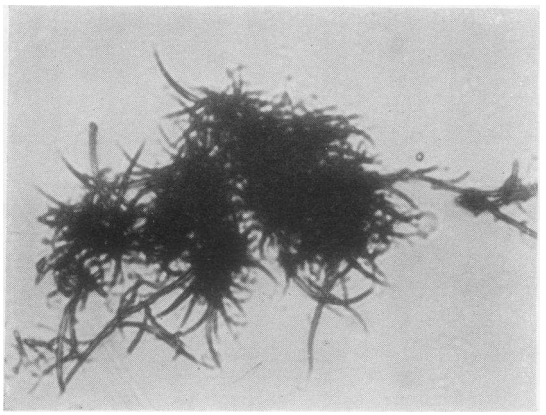

FIG. 1. CRYSTALS OF THE PORPHYRIN ESTER FROM THE URINE.

methyl ester of coproporphyrin I crystallizes characteristically in this form, i.e., thread like needles; while the crystals of coproporphyrin III methyl ester, as recently compared by H. Fischer and Duesberg (5), are less needle like, having the form of broader prisms. The porphyrin ester isolated in the above procedure, after three recrystallizations from chloroform-methyl alcohol, melted at 241 to 243° C. Coproporphyrin I methyl ester (for which the writer is indebted to Professor H. Fischer) melted at 245 to 246° C. A mixture showed no significant depression of melting point, which was 243 to 245° C. The absorption spectrum of the acetic and ether solution of the free porphyrin was as follows: I, 625.4 mμ to 621.4, maximum 623.5 mμ, II, very faint, maximum 596.8 mμ, III, 569.0 mμ to 567.1, maximum 568.2 mμ, IV, 532.8 mμ to 524.2, maximum 529.1 mμ, V, 505.2 mμ to 488.1, maximum 495.1 mμ. The order of intensity was: V, IV, I, III, II. This solution exhibited identical absorption with that of a similarly prepared solution of coproporphyrin I, the spectra of the two solutions being superimposed in the comparison spectrometer.

DISCUSSION

There was no suggestion in the behavior or appearance of the patient's erythrocytes, during life, of bone marrow irritation. Judging by Weir and Comfort's (17) recent review of the reported cases of cincophen poisoning, anemia or other evidence of marrow affection is extremely rare. There is little reason, therefore, to assume that this increase of coproporphyrin in the urine occurred because of increased formation in the marrow such as is the case in congenital porphyrinuria and pernicious anemia (H. Fischer, H. Hilmer *et al.* (18)). Rather than derived from excessive formation in the marrow it was believed that the porphyrin isolated was the normal coproporphyrin of the urine, increased because of the damaged excretory power of the liver. Van den Bergh (19) has recently discussed the retention of coproporphyrin in the blood serum in obstructive jaundice. He obtained experimental evidence to suggest that the coproporphyrin of the bile is formed in the liver. According to this, one would expect relatively large amounts of urinary coproporphyrin in simple obstructive jaundice, and smaller amounts or none in jaundice due to liver disease. This was apparently not true in the present case, since there was, of course, advanced liver disease. Nevertheless, the damage was fairly well limited to the periphery of the lobules, and there was obviously a definite biliary obstruction. In three cases of common duct obstruction due to stone, the urine was found to contain definite, but only moderate increases of coproporphyrin, very much less in amount than in the case of cincophen cirrhosis. On the other hand, the urine from a patient who later died of hepatic insufficiency due to advanced hepar lobatum contained no trace of either copro- or any other porphyrin.

SUMMARY

A porphyrin has been isolated from the urine of a patient with cirrhosis of the liver, the latter probably caused by cincophen. So far as can be determined this is the first report of the isolation of a porphyrin from the urine of a patient suffering from jaundice, or liver disease. It is believed to be the normal coproporphyrin of the urine, increased in amount because of damaged excretory power of the liver. It has been positively identified by virtue of ester melting point and spectroscopic character as coproporphyrin I.

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