CONCERNING THE NATURALLY OCCURRING PORPHYRINS

II. The Isolation of a Hitherto Undescribed Porphyrin Occurring with an Increased Amount of Coproporphyrin I in the Feces of a Case of Familial Hemolytic Jaundice [1]

By CECIL JAMES WATSON

(*From the Department of Medicine, University of Minnesota Hospital, Minneapolis*)

(Received for publication September 10, 1934)

Van den Bergh (19) has recently obtained experimental evidence to suggest that coproporphyrin may be formed in the liver from protoporphyrin. Having a short time previously described the regular occurrence of protoporphyrin in human erythrocytes (20) he pointed out the possibility that the coproporphyrin of human bile may have its origin in this way, i.e., protoporphyrin liberated from the red blood cells and transformed into coproporphyrin in the liver. The protoporphyrin of the erythrocytes has as yet received relatively little study. Van den Bergh's observation as to its occurrence has been confirmed by Kämmerer (10), and by Schreus (21). It has been assumed by Van den Bergh to correspond to aetioporphyrin III, in other words, to be related either to the formation or destruction of hemoglobin. Mention has been made in part I of the dualism of the porphyrins which H. Fischer has shown to exist in nature. A most complete review of this subject in its relation to physiology and medicine will be found in Kämmerer's presentation (10) before the German Congress of Internal Medicine in 1933.

If the coproporphyrin in the bile comes from the protoporphyrin of the erythrocytes, a considerable increase in its amount in both bile and feces might be expected during a period of increased blood destruction. Furthermore, if the protoporphyrin giving rise to coproporphyrin in the liver, does correspond to aetioporphyrin III, then the increased coproporphyrin excreted should be coproporphyrin III. For this reason the writer has examined the feces as to porphyrin content in five cases of congenital hemolytic jaundice. One of these was of particular interest since the patient was under observation during a so-called hemolytic crisis. The porphyrin excretion in the feces during this period was actually greatly increased. In the following, particular emphasis will be given to the description of the porphyrins isolated from the feces of this case.

Because of Boas' (22) recent description of the occurrence of protoporphyrin in the urine and his suggestion that it might be found in urines of individuals having increased blood destruction, the urine from this case was also carefully examined as to porphyrin content. Günther (12), later H. Fischer and Zerweck (9), and R. Duesberg (23) examined the urine in cases of hemolytic jaundice without being able to demonstrate any increase in porphyrin content.

MATERIAL AND METHODS

Case 1. Male, aged 22. The patient stated that he had never been strong and that for many years he had been subject to attacks of short duration characterized by nausea, vomiting, and fever. In the past three years jaundice had been noted repeatedly and anemia had been at times severe enough to require transfusion. The patient's mother believed that jaundice and anemia had been present in early childhood. The patient was first examined on November 21, 1933. At this time jaundice was moderate, and there appeared to be some anemia. He complained of weakness and tiredness; the mouth temperature was elevated to 99.4° F. The spleen was markedly enlarged, reaching to about the level of the umbilicus. Its consistency was firm. The hemoglobin percentage on this date was 54 (Sahli). The erythrocytes were 2,570,000; leukocytes were normal. Stained smears of the blood revealed marked anisocytosis with many hyperchromatic microcytes. The reticulated erythrocytes were 37 per cent. The icterus index was 20. The

[1] Aided by a grant from research funds of the Graduate School of the University of Minnesota.

Van den Bergh reaction was of the indirect type. Resistance of the patient's erythrocytes to hypotonic saline was as follows: hemolysis began at 0.66 per cent, was complete at 0.44 per cent. Hemolysis of control erythrocytes began at 0.44 per cent, was complete at 0.34 per cent. None of the members of the patient's immediate family, including his parents and several brothers, gave any history of jaundice, nor was the spleen palpable in any. Nevertheless, the erythrocytes from the father and one brother were tested as to fragility with interesting result. Father's erythrocytes: hemolysis began at 0.42 per cent and was complete at 0.34 per cent. Hemolysis of the brother's erythrocytes began at 0.54 per cent and was complete at 0.38 per cent. Control: Hemolysis began at 0.44 per cent, was complete at 0.32 per cent. The urobilinogen excretion in the feces was estimated over a four day period, November 21 to November 25. The average daily amount was 1140 mgm. The normal range with the method (13) used is from 100 to 250 mgm. per day. Preparatory to splenectomy the patient was given a transfusion of 600 cc. of citrated blood on November 26. It should be stated that even before the transfusion the patient complained of weakness and anorexia and the temperature was elevated to 100° F. There was no immediate reaction to transfusion. On the following day the patient felt much worse, the temperature rose to 103°, nausea, restlessness, and apprehension were noted. The jaundice had increased. On November 29 the icterus index had increased to 32, the hemoglobin percentage had decreased to 45 (Sahli). The average daily excretion of urobilinogen in the feces from November 28 to December 2 was 1220 mgm. It was believed that the patient was suffering from a hemolytic crisis, and that the transfusion rather than having been of aid had perhaps precipitated this increase of blood destruction. Following this temporary marked increase of jaundice and anemia, improvement was rapid. The temperature was normal on December 1 and the patient felt much better. After three days he was allowed to go home and returned on December 12. At this time he had improved remarkably. The icterus index was 20, jaundice definitely diminished. The hemoglobin percentage was now 78 (Sahli), and the erythrocytes were 4,200,000 per c.mm. The reticulocytes had decreased to 9 per cent. Splenectomy was done by Dr. O. H. Wangensteen. The spleen weighed 1720 grams. The capsule was smooth, the external surface was dark red, the consistency firm. The cut section was dark red, and it was apparent that the pulp contained a large amount of blood. Microscopic sections exhibited the usual appearance of spleens from cases of familial hemolytic icterus, i.e., narrow sinuses, the splenic pulp crowded with erythrocytes. Relatively little iron was demonstrable with the Berlin blue reaction. The spleen was not examined as to porphyrin content. This had previously been done in a similar instance (24) with negative result.

The collection of feces from which porphyrins were isolated covered two periods, November 21 to November 25, and November 28 to December 2, eight days in all. The moist feces for this period weighed 920 grams. Twenty grams were used in the quantitative urobilinogen estimations. One gram of the mixed collection was used for a benzidine test for occult blood, which was negative.

All of the remainder was thoroughly ground in a large mortar with glacial acetic acid. This mixture was repeatedly extracted with relatively larger amounts of ether, in the same way as recently described (25) for the isolation of crystalline stercobilin from feces. The acetic ether extract was filtered and the ether largely removed by vacuum distillation. The remaining acetic acid solution was poured into 6 volumes of 2 per cent HCl, with thorough mixing. After standing over night this was filtered, and the filtrate in a five liter separatory funnel was covered with approximately 500 cc. of ether. Solid sodium acetate was now added in sufficient amount to make the aqueous fraction negative to congo paper. The two fractions were thoroughly shaken immediately after each addition of sodium acetate. After making the solution congo negative, it was shaken out four more times with smaller amounts of ether. The entire ether solution, containing all of the porphyrins, a considerable proportion of the copromesobiliviolin (26), and a relatively small proportion of the stercobilin, was washed repeatedly with distilled water, which removed most of the latter. The porphyrins and the copromesobiliviolin were removed by repeated ex-

traction with 2 per cent HCl. The copromesobiliviolin was removed from this by repeated extraction with chloroform. The porphyrins, with the exception of protoporphyrin, remain in the 2 per cent HCl.

The 2 per cent HCl solution was diluted with distilled water to 0.2 per cent. Following this dilution chloroform extraction was again resorted to. H. Fischer (27) was able to show that coproporphyrin may be separated from deuteroporphyrin by repeatedly extracting a 0.2 per cent HCl solution of the two porphyrins with chloroform. Only the deuteroporphyrin goes into the chloroform. This method was carried out by the writer (24) in isolating deuteroporphyrin III from the feces.

The chloroform solution had a wine red color and exhibited intense red fluorescence in ultraviolet light, indicating a considerable porphyrin content. It was shaken repeatedly in a separatory funnel with 10 per cent NaOH, the porphyrin leaving the chloroform quantitatively as a difficultly soluble sodium salt. On standing over night, most of this came out of solution. It was collected on a filter paper, washed with a very little 10 per cent NaOH, and then redissolved in 10 per cent HCl. It was then taken into ether by repeated extraction, the HCl being almost neutralized by the gradual addition of 10 per cent NaOH. The ether solution was dried over anhydrous sodium sulphate, filtered and evaporated to dryness on the water bath. The partially crystalline residue was dissolved in methyl alcohol saturated in the cold with dry HCl gas. The purified ester was crystallized out of chloroform-methyl alcohol. All of these procedures were carried out in accordance with H. Fischer's (4, 16) methods. After three recrystallizations, the yield was approximately 7 mgm. As will be seen below, this was found to be a hitherto undescribed porphyrin.

The porphyrin remaining in the 0.2 per cent HCl was taken into ether after making the solution negative to congo paper by the addition of sodium acetate and more strongly acid by the addition of glacial acetic acid. After washing the ether several times with distilled water, the porphyrin was extracted with 10 per cent NaOH. The sodium salt of this porphyrin was soluble. The solution was filtered and the porphyrin taken into ether by barely acidifying with 10 per cent HCl and shaking in a separatory funnel. The ether was washed with water and the porphyrin quantitatively extracted with 1 per cent HCl. This fractionation: ether—1 per cent HCl—ether was repeated, just neutralizing the acid each time by dropping in 10 per cent NaOH. The ether was dried over anhydrous sodium sulphate. Upon concentrating it to a very small volume on the water bath, the free porphyrin crystallized in fine needles. The small amount of solution was filtered and all of the crystalline material including that on the walls of the round bottomed flask was esterified as described above. The ester crystallized readily from chloroform-methyl alcohol. The yield was approximately 6 mgm. As will be described below this was found to be the methyl ester of coproporphyrin I.

For the purpose of spectroscopic study small amounts of the esters were saponified by standing over night in 25 per cent HCl. This was made negative to congo paper, and the free porphyrins extracted with ether. Measurements of absorption spectra were made with the aid of a Zeiss grating comparison spectrometer. Comparisons of spectra of known and newly isolated porphyrins were made by superimposing the spectra of acetic and ether solutions of the two in this apparatus. In this way it is possible to make certain of very small differences in position of absorption bands. These differences are often too small to be recognized with a hand spectroscope.

Coproporphyrin was demonstrated in the urine of Case 1 by employing the acetic and ether extraction method as used for the isolation of coproporphyrin I from the urine, described in Part I. The amount obtained from a 24-hour urine sample was too small to color more than faintly the final solution in 2 per cent HCl, 20 cc. in volume. This, however, exhibited a fairly intense red fluorescence in ultra-violet light. The amount of porphyrin was too small to permit of spectroscopic identification. It was ether soluble and did not leave 0.2 per cent HCl for chloroform, characteristics which identify it fairly well as a coproporphyrin. Two months after splenectomy feces from Case 1 for an eight day period of collection, were again examined, using the method just described. At this time there was no jaundice nor anemia and the patient felt very much improved.

Feces for eight day periods of collection from four other typical cases of familial hemolytic anemia were also investigated as to porphyrin content. The method described above for isolating porphyrins from the feces was used in each instance. In three of the cases (3, 4, and 5), no complicating disease was present. In one (Case 2), chronic ulcerative colitis had been recognized for two years; during the period of collection of feces the patient was having from four to six stools per day. These at times contained traces of blood. In none of these four cases was there any evidence of a hemolytic crisis such as occurred in Case 1. In this study, no attempt has been made to estimate the amounts of porphyrin excreted, principally because no accurate and satisfactory method is yet available. Nevertheless, it was possible to gain what was believed to be a fairly accurate conception of amount by the amounts which could be isolated, as well as by comparisons of the intensity of color and absorption spectrum of the final solutions from amounts of feces representing the same periods of collection. The cases used for comparison will be mentioned in Part III of this communication.

RESULTS

The methyl ester of the chloroform soluble porphyrin obtained from the feces of Case 1 crystallized in flower-like aggregates of well formed, somewhat curved prisms (Figure 1). The ease of crystallization, as well as the unity of the crystal form spoke against the possibility of a mixture.

This ester melted sharply at 202 to 203° C. There was no elevation of the melting point after another recrystallization. The dimethyl ester of deuteroporphyrin III melts at 223°, that of mesoporphyrin IX at 212°, that of hematoporphyrin at 212° (H. Fischer (2)). Mixed melting point determinations with deuteroporphyrin III and mesoporphyrin IX ester, the latter obtained from hemin according to H. Fischer and Kögl's method (28), gave sharp depressions.

The "HCl number" (2) of the new porphyrin was found to be 0.8 per cent. This indicates the lowest concentration of HCl which will remove the porphyrin from ether. The absorption spectrum of this porphyrin in ether and acetic was: I 630.1 to 621.9 mμ, maximum 627.4 mμ, II (very weak) 597.2 mμ maximum, III 582.8 to 566.7 mμ, maximum 572.4 mμ, IV 536.4 to 525.0 mμ, maximum 531.4 mμ, V 506.6 to 486.4 mμ, maximum 496.9 mμ. Order of intensity: V, IV, III, I, II. A small amount of this porphyrin was submitted to Professor Hans Fischer in Munich, to whom the writer is most grateful for suggestions and advice regarding it. Professor Fischer found: I 628.9 mμ, maximum, II 572.3 mμ, III 532.9 mμ, IV 499.4 mμ; order of intensity IV, III, II, I. He observed that this was not characteristic either of hematoporphyrin or deuteroporphyrin, but more like a mixture of protoporphyrin and hematoporphyrin. Because of this he pointed to the possibility of a molecular combination of the two. Another possibility which he suggested was that of a protoporphyrin changed to the extent of having a molecule of water added on to one of its vinyl ($CH=CH_2$) groups. Hematoporphyrin in concentrated sulphuric acid exhibits a marked spectroscopic change compared with the original substance; deuteroporphyrin shows no change (27). The writer did not observe any change in concentrated sulphuric acid after one hour. However, Professor Fischer noted a definite although slight shift of absorption after the solution had stood for three days. His spectroscopic findings were: I 597.5 mμ, II 552.8 mμ. After three days I 596.5 mμ, II 558.8 mμ. Deuteroporphyrin is readily brominated giving dibromdeuteroporphyrin with considerable change in spectroscopic character, while mesoporphyrin is unchanged by bromine. The new porphyrin exhibited a very definite change with bromine, and

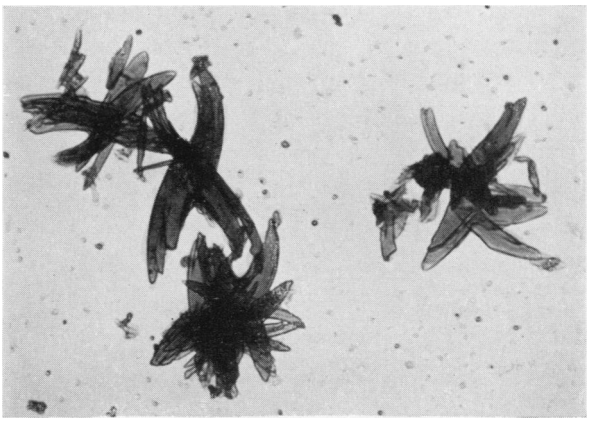

FIG. 1. CRYSTALS OF PORPHYRIN ESTER FROM THE FECES IN CASE 1.

the absorption was now identical with that of dibromdeuteroporphyrin III. The effect of bromine was observed with the free porphyrin dissolved in glacial acetic acid. Deuteroporphyrins corresponding to aetioporphyrin I have not been synthesized (27), so that no reference substance is available to aid in determining whether the new porphyrin is an isomeric deuteroporphyrin, or an entirely new type of porphyrin. Microanalysis would be of great help in classifying this porphyrin. To this end more material is necessary, and feces from more individuals exhibiting a marked increase of blood destruction must be studied as to porphyrin content. The feces from Case 1 obtained two months after splenectomy contained only a trace of chloroform soluble porphyrin. The amount was too small to be identified spectroscopically.

In three of the four other cases studied (2, 3, and 4), the amounts of chloroform soluble porphyrin were not sufficient for identification. From the feces of Case 5, however, a chloroform soluble porphyrin was obtained in sufficient concentration to permit spectroscopic study. In ether and acetic the absorption spectrum was: I 630.8 to 621.8 mμ, maximum 627.4 mμ, II 582.8 to 567.3 mμ, maximum 576.2 mμ, III 538.6 to 526.1 mμ, maximum 533.0, IV 509.2 to 491.7 mμ, maximum 498.3 mμ. Order of intensity: IV, III, I, II. Superimposed with the new porphyrin from Case 1, the absorption spectra were identical. Thus the occurrence of the newly described porphyrin in the feces of another case of familial hemolytic jaundice, while not proven, is strongly suggested.

The ester of the porphyrin remaining in 0.2 per cent HCl, from Case 1, crystallized in the form of thread like needles, characteristic of coproporphyrin I ester (see Figure 1 in Part I). This melted at 245 to 246° C. The melting point of coproporphyrin I methyl ester was identical. A mixture gave no depression of the melting point. In acetic and ether the absorption spectrum was: I 625.4 to 621.4 mμ, maximum 623.5 mμ, II (very faint) maximum at 596.8 mμ, III 569.0 to 567.1 mμ, maximum 568.2 mμ, IV 532.8 to 524.2 mμ, maximum 529.1 mμ, V 505.2 to 488.1 mμ, maximum 495.1 mμ. Order of intensity: V, IV, I, III, II. Spectroscopic identity with coproporphyrin I was demonstrated, the spectra being superimposed.

Roughly estimated, the per diem amount of coproporphyrin I excreted by Case 1, before splenectomy, was at least 20 to 30 times that normally observed. This was much more than was found in the other four cases, although the amount of coproporphyrin in the feces of each of these was definitely greater than from normal individuals and individuals with secondary anemias. The cases used for comparison will be mentioned in Part III of this communication. Coproporphyrin I was readily isolated from the feces of Cases 3 and 5; in the former the amount obtained after three recrystallizations was approximately 2 mgm., in the latter 3 mgm. The melting points in each instance were identical with that of coproporphyrin I, and no depression of the melting point of a mixture was observed. The absorption spectra in both instances were identical with that of coproporphyrin I, the spectra being superimposed. The amount of porphyrin from Case 4 was easily sufficient to permit crystallization, and was at least as much as in the last two instances, but it was unfortunately lost during recrystallization. The amount from Case 2 was not enough to be obtained in crystalline form, although the intensity of color and absorption spectrum indicated considerably more porphyrin than usually noted.

DISCUSSION

In only one way would it be possible to correlate the occurrence of excessive amounts of coproporphyrin I in the feces during heightened blood destruction with Van den Bergh's conception that the protoporphyrin of the erythrocytes is transformed in the liver into coproporphyrin. This would presuppose that this protoporphyrin also corresponds to aetioporphyrin I, in other words, that it has no direct relation to the formation or destruction of hemoglobin. The only protoporphyrin as yet isolated is that whose iron chloride compound is ordinary hemin, whose structure corresponds to aetioporphyrin III. Nevertheless, it is quite possible that isomeric protoporphyrins exist in nature, and that the formation of coproporphyrin I in the body proceeds over a protoporphyrin of the "I" type. H. Fischer and Kirrman (15) have shown that protoporphyrin may be con-

verted to coproporphyrin by addition of formic acid to the two vinyl groups of the former. These relationships have been thoroughly presented by Fischer in various communications (1, 2, 3).

Until the isomeric type of the new porphyrin can be determined, little can be said regarding its significance. Its characteristics classify it rather definitely as a closer relative of protoporphyrin than is coproporphyrin. If new evidence shall prove it to correspond to aetioporphyrin III, then its occurrence during heightened blood destruction could only be regarded as indicating biliary excretion of a porphyrin derived from hemoglobin. If it be found to correspond to aetioporphyrin I, its occurrence during increased blood destruction would strongly suggest that the erythrocytes contain a protoporphyrin corresponding to aetioporphyrin I.

The possibility that these porphyrins were formed in the bone marrow, not in the liver, must be considered. Against this view was the very small amount of coproporphyrin, and the total absence of a chloroform soluble porphyrin, in the urine of Case 1. A microspectroscopic study of the marrow in familial hemolytic jaundice such as Borst and Königsdörffer (29) have carried out in pernicious anemia, would serve to support or deny the possibility of bone marrow formation.

SUMMARY

A hitherto undescribed porphyrin whose methyl ester melts at 202 to 203° C., having some of the characteristics of a deuteroporphyrin, but differing from the deuteroporphyrins spectroscopically, has been isolated from the feces of a typical case of familial hemolytic jaundice, suffering from a " hemolytic crisis " at the time the feces were collected. This porphyrin occurred in association with a marked increase of coproporphyrin I. In four other cases of the same disease the excretion of coproporphyrin was moderately increased, and in two of these instances it was again isolated and shown to be coproporphyrin I. If this coproporphyrin is to be related to the protoporphyrin of the erythrocytes, as described by Van den Bergh, the latter would have to correspond to aetioporphyrin I. The possibility of independent formation in the marrow erythroblasts must also be considered.

BIBLIOGRAPHY

See this issue, page 109.