[CONTRIBUTION FROM THE C. F. KETTERING FOUNDATION FOR THE STUDY OF CHLOROPHYLL AND PHOTOSYNTHESIS]

Occurrence of Decomposition Products of Chlorophyll. III.[1] Isolation of Pyrroporphyrin from Beef Bile[1a]

BY PAUL ROTHEMUND

Since the discovery of the presence of phylloerythrin in beef bile by Löbisch and Fischler,[2] bile has been the source for the preparation of this decomposition product of chlorophyll. The original method of isolation has been improved by different authors[3] and leads to a beautifully crystallized product. Depending upon the method used, one obtains phylloerythrin either in form of $C_{33}H_{34}N_4O_3$, or with one molecule of chloroform of crystallization for every two molecules of phylloerythrin, $(C_{33}H_{34}N_4O_3)_2CHCl_3$. Synthetic phylloerythrin was first prepared by Fischer,[4] but beef bile is still the best source for its preparation, due to the low cost and the ready availability of this starting material.

Using the methods referred to above, large quantities of bile were worked up in our laboratory for the preparation of phylloerythrin. The data of the elementary analyses, as reported in the literature for the product thus prepared, as well as the analyses of our product, showed small but consistent deviations from the theoretical values for phylloerythrin. In some cases, the examination of a solution in pyridine–ether with the Zeiss pocket spectroscope with wave length scale and comparison prism, revealed a faint but distinct absorption band at approximately 625 mμ.

Some time ago, we had demonstrated the occurrence of a number of chlorophyll derivatives in the stomach contents, in the stomach walls, and in the digestive system of herbivorous animals;[5] the presence of other substances of porphyrin character besides phylloerythrin in beef bile could, therefore, be assumed, and the spectroscopic finding proved this assumption to be correct. The method of fractionation of an ether solution of the bile pigments with hydrochloric acid was used for the separation.

Coproporphyrin, which is a physiological constituent of *human* bile[6] and is formed there from the protoporphyrin of the red blood cell,[7] was found in the 0.5% hydrochloric acid fraction only in traces. This finding is interesting in the light of the fact that coproporphyrin can always be detected spectroscopically in cow's milk; the volume of bile necessary to obtain a positive test for this substance is much larger than that of milk.

The chlorophyll porphyrins phyllo- and rhodoporphyrin could not be found in bile; it was, however, possible to demonstrate the presence of pyrroporphyrin. This porphyrin was responsible for the spectroscopic finding mentioned above; the position of its main red absorption band, measured on a large grating spectroscope, is 623.1 mμ in ether. It was isolated from the 3% hydrochloric acid fraction and analyzed. The methyl ester was prepared.

The 10% hydrochloric acid fraction yielded phylloerythrin in approximately the same quantity as obtained by the chloroform/pyridine–alcohol method in which fractionation is omitted.

Two fractions were separated using higher concentrations, 15 and 20% hydrochloric acid, but the quantity of pigment in each of these fractions was too small to permit the isolation. The absorption spectra of these two fractions in ether are given in the experimental part.

Experimental

Twelve liters of beef bile was evaporated to a volume of 1.5 liters, and transferred into a 5-liter separatory funnel by means of 750 cc. of water. After acidification with 10 cc. of concd. hydrochloric acid and thorough shaking, the bile was extracted with a mixture of 1800 cc. of chloroform and 300 cc. of pyridine. The chloroform–pyridine layer was filtered and the chloroform distilled off. To the recovered chloroform about one-tenth of its volume of pyridine was added, and a second extraction of the bile solution was performed with this mixture. The extract was added to the residue from the first extraction, and the chloroform recovered. The combined material from six extractions was concentrated *in vacuo* to a volume of 150 cc., and transferred into 2 liters of ether by means of 200

(1) Paper II, THIS JOURNAL, **56**, 2400 (1934).

(1a) This investigation was carried out with the experimental cooperation of Miss Eleanor Bates and Mr. Charles Roland.

(2) Löbisch and Fischler, *Monatsh.*, **24**, 335 (1903).

(3) Fischer and Bäumler, *Ann.*, **474**, 65 (1929); **480**, 197 (1930); Marchlewski, *Z. physiol. Chem.*, **185**, 8 (1929); *Bull. Acad. Polon. Sciences*, **Ser. A**, 599 (1929); Noack and Kiessling, *Z. physiol. Chem.*, **182**, 13 (1929); **193**, 97 (1930).

(4) Fischer and Riedmair, *Ann.*, **497**, 181 (1932).

(5) Rothemund and Inman, THIS JOURNAL, **54**, 4702 (1932); Rothemund, McNary and Inman, *ibid.*, **56**, 2400 (1934).

(6) Schumm, *Z. physiol. Chem.*, **153**, 225 (1926).

(7) Hijmans van den Bergh, *Nederland. Tijdschr. Geneeskunde*, **76, I**, 120 (1932); Hijmans van den Bergh, Grotepass and Revers, *Klin. Wochschr.*, **11**, 1534 (1932).

cc. of pyridine; 500 cc. of water was added, and shaken, until the precipitate was dissolved. The water layer was separated, diluted with 500 cc. of water, and extracted with 250 cc. of ether. The combined ether solutions were washed with 150-cc. portions of water, until the water layer showed only slightly yellow color (3 to 6 washings were usually sufficient). The dark red-brown ether solution was subjected to fractionation with hydrochloric acid by Willstätter's method.[8]

0.5% Hydrochloric acid was used in seven portions of 100 cc. each for the first fraction. The pigment from the combined extracts was transferred into 500 cc. of ether by means of concd. sodium acetate solution. This ether was washed acid free and evaporated to a small volume; the coproporphyrin was refractionated with 0.1% hydrochloric acid and identified spectroscopically only. Fifty liters of bile did not yield enough material for esterification and determination of the melting point of the ester.

Ten 100-cc. portions of the 3% hydrochloric acid fraction were combined, the pigment transferred into ether in the usual manner, the ether washed and refractionated with 1.5% hydrochloric acid. The porphyrin was brought back into ether with sodium acetate. Direct spectroscopic comparison with an ether solution of pyrroporphyrin from pheophytin showed identity. The hydrochloric acid number was 1.5. The porphyrin was quantitatively removed from the ether by means of 0.1 *N* potassium hydroxide or a 10% sodium carbonate solution. Esterification with methanolic hydrochloric acid yielded pyrroporphyrin methyl ester of m. p. 241°, spectroscopically identical with the free porphyrin. No depression of the melting point was observed with pyrroporphyrin ester prepared from pheophytin.

Anal. Micro Kjeldahl determinations: pyrroporphyrin (material from 100 l. of bile recrystallized from pyridine–methanol). Calcd. for $C_{31}H_{34}N_4O_2$: N_2, 11.34%. Found: N_2, 11.2%. Pyrroporphyrin methyl ester (recrystallized from pyridine–methanol). Calcd. for $C_{32}H_{36}N_4O_2$: N_2, 11.02. Found: N_2, 10.9.

Phylloerythrin was extracted from the ether solution with 10% hydrochloric acid. In addition to the normal absorption bands of phylloerythrin, a weak band at 656.0 $m\mu$ was observed in the ether solution of phylloerythrin, which had been subjected to hydrochloric acid fractionation. The substance to which this band is due, remained in the mother liquid, when the phylloerythrin was recrystallized from pyridine.

The 15% hydrochloric acid fraction was transferred to ether and exhibited then the following absorption bands: I, 664.4–(656.6)–648.8---635.7; II, 608.0---597.0–(592.3)–587.5---580.0; III, 566.0–(555.7)–545.4; IV, 534.8–(530.8)–526.8; V, 507.2–(499.2)–491.1; E. A. 465. Intensity: I, II, III, IV, V.

The spectrum of the 20% hydrochloric acid fraction in ether was: I, 601.6–(589.3)–577.0; II, 563.4–(558.5)–553.7; III, weak band with its maximum approximately at 521; E. A. 442. Intensity: I, II, III.

(8) Willstätter and Mieg, *Ann.*, **350**, 1 (1906).

Summary

1. Pyrroporphyrin was found in beef bile under physiological conditions; its quantity changed only very slightly with the season of the year. Traces of coproporphyrin were also always present.

2. The separation from phylloerythrin, and from other pigments in bile, was performed by fractionation of an ether extract of the pigments with hydrochloric acid.

3. It is assumed that the occurrence of pyrroporphyrin in beef bile is due to decomposition of chlorophyll, while the presence of coproporphyrin is accounted for by the decomposition of hemoglobin.

4. No evidence for the presence in bile of other chlorophyll porphyrins (phyllo- or rhodoporphyrin) could be obtained; neither was it possible to demonstrate the occurrence of other porphyrins from blood pigment (*e. g.*, proto- or deuteroporphyrin).

ANTIOCH COLLEGE
YELLOW SPRINGS, OHIO

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