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Some Cases of Congenital Porphyrinuria in Cattle: Chemical Studies upon the Living Animals and Post-Mortem Material.

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SEVERAL living bovine cases of chronic Porphyry or congenital Porphyrinuria were recently encountered on a farm near Bremersdorp in Swaziland. An account of the discovery of these cases and of the clicinal picture they present is being submitted by Fourie (1936). The condition, which also occurs in human beings is extremely rare and for this reason the opportunity of working with living animal cases is to be considered very fortunate. Fourie has reviewed existing knowledge of the disease from its clinical aspect and has also stressed the difference between true porphyry and " ochronosis " where the pigment deposited in the bones appears to have the character of a melanin. Congenital porphyrinuria would seem to be an inherited metabolic anomaly comparable in some respects with alcaptonuria (Garrod, 1923); however, the meagre data available in records from human and veterinary medicine is insufficient to do more than lend a suggestion of the hereditary factor in the transmission of the disease. The present cases include both males and females, all the progeny of a single pure-bred shorthorn bull. This bull, together with a few normal heifers he has sired and normal cows which have produced affected calves, is at present at Onderstepoort and will be used for breeding purposes.* Controls will be drawn from the Laboratory herd.

Since Fourie intends to discuss the disease from a general standpoint, it is proposed to confine this article to the strictly chemical aspect and to record the chemical examination of one case which was slaughtered for the purpose of investigation. Blood, urine, faeces, bile, bones, bone-marrow, liver, spleen and kidneys were examined. The pathology will be considered in a later paper by one of the collaborators in this investigation.

* On examination, the animals were found to be suffering from bilharziosis. They are being treated for this condition before accurate clinical studies are made. Bilharzia urines which I have examined have, however, contained no more than the usual trace of coproporhyrin (see Appendix).

Of the 15 or so human cases of porphyry which have been noted (compare Günther 1925) only one, that of the man Petry, has been studied in any detail and, with the exception of an isolated observation, that an ox prior to slaughter " was supposed to have passed blood-stained urine " (Schenk 1902) no record of a living animal case exists. Upon Petry's death, his bones, organs, blood, bile, etc., were examined chemically by Fischer, Hilmer, Lindner and Pützer (1925) and the histo-pathological examination carried out by Borst and Königsdörfer (1929) who utilised for this purpose the technique of ultra-violet fluorescence microscopy and published their results in a classical monograph.

Perusal of the literature shows that a chocolate colouration of the bones of slaughtered animals has been observed by Mettam (1910), Witte 1913), Schmey (1913 a; 1913 b), and Maraev (1928). Schmey (1913, b) and Poulsen (1910) were among the first to recognise this condition as an entity distinct from " ochronosis " a term introduced originally by Virchow in 1866 with reference to human material in which the pigment was subsequently proved to be of melanin nature.

The bone pigment of animal porphyry was first identified as uroporphyrin by Fink (1931). The case was that of a bovine and the only material available was 19 gm. of rib, 23 gm. of vertebrae and 5 gm. of bone marrow, together with a piece of kidney. Neither marrow nor kidney yielded any result. The pathological examination of the case was carried out by Fikentscher (1931) who found some lamellae of the bones, both periosteum and endosteum, to be stained by a diffuse brown pigment exhibiting in ultra-violet light a rosy fluorescence with the following spectral bands:—

692; 670-647; 632-614; 596-575.

These figures correspond with the ultra-violet emission spectrum of uroporphyrin as determined by Borst and Königsdörfer.

Shortly afterwards, a further case was encountered in the Leipsig abattoir and was studied by Fink and Hoerburger (1931) who succeeded in crystallising the uroporphyrin ester (M.P. 293°) from the bones (15 mgm. from 630 gm. bones) and identifying it as uroporphyrin I by comparison with a specimen originally isolated from Petry urine. They employed for the comparison the elegant pH-fluorescence technique developed by Fink and Hoerburger (1933 a, 1933 b, 1934 a, 1935 a, also Hoerburger 1933).

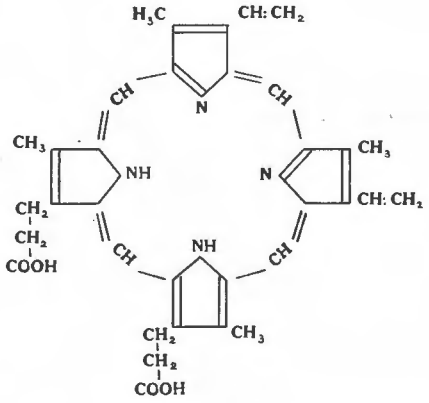
Yet another case has been reported upon during the past year (Fink and Hoerburger 1935 b) and the appearance of concentric rings of lighter and darker colour in a cross section of the bone described. This feature had not previously been encountered by Fink and Hoerburger but it may be stated at once that in skeletal material from both the cases which have been studied during the course of the present work, similar annular zones of pigment were very clearly defined. They most probably represent accumulations of pigment deposits during periods of arrested bone growth rather than periodic variations in the intensity of the disease.

Up to the time that the present studies were undertaken, no examination of the urine, faeces or organs of affected animals had been made nor was any report upon the clinical condition available.

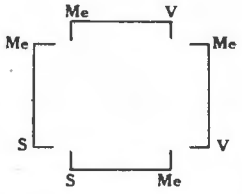
THE NATURAL PORPHYRINS.

In order to illustrate the following discussion of the experimental findings, it is felt advisable to present a brief summary of the chemistry of the porphyrins, their configurational and isomeric relationships and the present state of knowledge concerning their occurrence in nature [for general reviews see Fischer (1931), Kämmerer (1933)].

By removal of iron from haematin, is obtained the substance protoporphyrin (Fig. 1), a complex of four pyrrole nuclei united by methine bridges -CH= and possessing, as substituents, four methyl groups CH_3, two vinyl groups $-CH=CH_2$ and two propionic acid groups; $-CH_2 \cdot CH_2 \cdot COOH$, in the positions shown. A convenient shorthand method for writing such formulae has been introduced by Fischer in which only the pyrrole rings are represented and these merely by the sign  the substituents X and Y being in the $\beta\beta'$ positions. A reference to the example of protoporphyrin will make this clear (see Fig. 2).



Protoporphyrin III



Protoporphyrin (abbreviated)

Figs. 1 and 2.—Protoporphyrin III

Key:

Me = CH_3.
V = $CH=CH_2$.
S = $CH_2 \cdot CH_2 \cdot COOH$.

Inspection of the formula for protoporphyrin will show that numerous isomeric modifications are possible depending upon the way in which the different substituent groups are arranged. This number becomes reduced to four in the case of aetioporphyrin, a substance obtainable from protoporphyrin, in which the substituent groups are 4 ethyl and 4 methyl groups only. The four aetioporphyrins, aetioporphyrin I, II, III, and IV (see Fig 3) can thus be regarded as the starting points of four series of derivatives. Actually, those belonging to the series II and IV have so far not been encountered in nature so that they can, for all practical purposes be dropped from this discussion. There remain the members of the I and III series.

H. Fischer and his collaborators have shown in a brilliant series of researches, culminating with the synthesis of haematin (Fischer and Zeile 1929; Fischer 1929) that both haemoglobin and chlorophyll belong to series III, whereas the porphyrins encountered in disease frequently belong to series I.

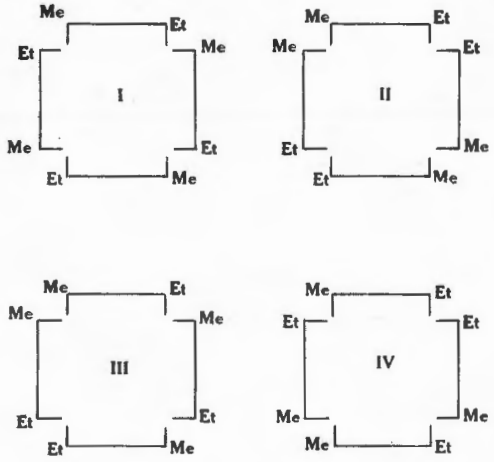


Fig. 3.—The four Aetioporphyrins.

The distinction is important, since it will be realised that the assignation of any particular pigment to the series I at once precludes its formation from normal haemoglobin by breakdown. Passage from one series to the other could only be effected by complete dissolution of the structure into its constituent pyrrole units and resynthesis in the new configuration.

Protoporphyrin, as already pointed out, possesses two carboxyl and two vinyl groups. On replacement of the latter by propionic acid residues, a tetra-carboxylic porphyrin would result. This is, in fact, the structure of the coproporphyrins (for synthesis see Fisher and Andersag 1926, and Fisher, Platz and Morgenroth, 1929), whilst uroporphyrin possesses, in all, eight carboxyl groups, arranged as can be seen by reference to Figs. 4 and 5. Since the free porphyrins do not possess sharp melting points, they are usually converted into their crystalline methyl esters for identification.

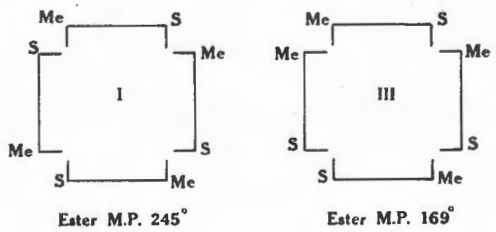
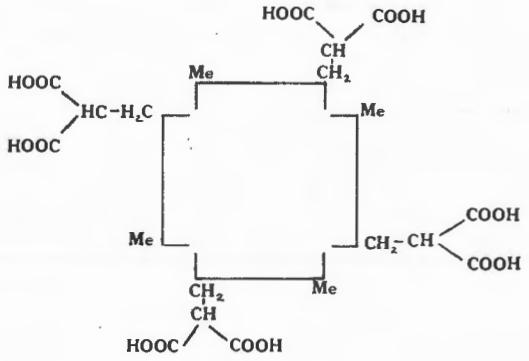


Fig. 4.—Coproporphyrins I and III.



Uroporphyrin I.

Fig. 5.—Uroporphyrin I.

Occurrence and Distribution of the Porphyrins.

The porphyrins are fairly widely distributed in nature, thus small quantities of coproporphyrin occur in normal urine and faeces (Fischer, 1915/16, Garrod, 1892, Fischer and Zerweck, 1924 a), in yeast, plants (Fischer and Hilger, 1924, a) and other materials, whilst the pigment of the tail feathers of the Turaco (*Turacus corythaix*) is the copper complex of uroporphyrin (Church, 1869; Fischer and Hilger, 1924 b) and the colouring matter of the egg-shells of many birds has been shown to be protoporphyrin [= öoporphyrin = Kämmerer's porphyrin] (Fischer and Kögl, 1923; Fischer and Kögl, 1924).

Thanks to the synthetic work of Fischer and his associates, we are now in a position to assign most natural porphyrins, if isolated in sufficient quantity, to their respective isomeric series, by comparison of the ester melting points with authentic material. The importance of thus distinguishing between the haemoglobin or III series pigments and the I series can not be over-emphasised as without this knowledge, speculation as to the mode of origin or function of any pigment is groundless.

To attempt a complete survey of the occurrence of porphyrins would be too lengthy a task, consequently only the most important data will be mentioned here.

Uroporphyrin was first isolated by Fischer (1915; Fischer and Zerweck, 1924, b) from the urine of the porphyrinuric patient Petry and characterised as an octa-carboxylic acid. From the faeces of the same case a porphyrin with four carboxyl groups, coproporphyrin was isolated (Fischer, 1915/16). It occurred in smaller quantities together with uroporphyrin in the urine (Fischer, 1916, a). These two pigments have since been shown to belong to the I series, and to be present in other cases of congenital porphyry.

Even in normal urine and faeces (Schumm, 1923; Fischer and Zerweck, 1924, a, 1924, c) small quantities of coproporphyrin are known to occur.

Fink and Hoerburger (1934, b) have performed the signal service of characterising the urinary constituent as coproporphyrin I.

The normal excretion of an unphysiological coproporphyrin which can not be derived from haemoglobin breakdown, is at first sight difficult to explain. It might be thought that it was derived from disintegrated cytochrome or catalase molecules but the evidence available to date indicates that the porphyrins derivable from the latter substances belong to the III series (Stern, 1935; Zeile and Reuter, 1933). Van den Bergh, Grotepas and Revers (1932) demonstrated the existence of small quantities of protoporphyrin in the red blood cells of healthy human subjects [confirmed by Kämmerer (1933 and Schreus (1934)] and were also able to show that added protoporphyrin, in liver perfusion experiments, was converted, in part, into coproporphyrin, eliminated via the bile. They were clearly of the opinion at the time that the protoporphyrin of the red cells is protoporphyrin III and that the biliary and urinary coproporphyrins belonged to the same series. No evidence is available which would assign the protoporphyrin of the erythrocytes to either series and to the writer it seems much more probable, in view of recent developments, that it will ultimately prove to be a series I pigment. The relationship between it and the urinary porphyrin would then become clear and the Van den Bergh experiments fall into their logical place.

A clue is perhaps to be sought in the examination of foetal blood and this it is hoped to do at this Laboratory. According to Finkentscher (1935), the serum of the normal human foetus at the 4th or 5th month contains 8-10 γ per 100 c.c. of an ether-soluble porphyrin resembling coproporphyrin, the quantity slowly decreasing to 1-3 γ at the time of birth. Subsequently, as shown by Herold (1934), there is, during the first five or six days of extrauterine life, a pronounced excretion of porphyrin by the infant, possibly to be correlated with the extensive breakdown of red cells which then occurs (compare Volhard, 1930). Haurowitz (1935) has shown that there is a foetal type of haemoglobin, different in crystalline form and other properties from normal adult haemoglobin and it seems to the writer of the greatest importance to ascertain whether the pigment moiety belongs to the I or III series. This finding in conjunction with the study of the foetal porphyrins and those of pernicious anaemia and congenital porphyrinuria might help to elucidate the whole problem of haemoglobin synthesis and metabolism and afford evidence against or in support of the oft-expressed view (compare Ehrlich, 1892; Duesberg 1931) that in the two diseases mentioned the organism exhibits an atavistic tendency so far as its pigment metabolism is concerned. Watson (1935, a) has already shown that coproporphyrin I is eliminated in the faeces of pernicious anaemia cases but disappears following liver therapy.

Certain states of intoxication in the adult are followed by excretion of porphyrin and the evidence is gradually accumulating to show that the pigments, in this instance, frequently, although not invariably belong to the III or normal haemoglobin series. Thus, Grotepass (1932) identified the coproporphyrin present in the urine

after lead poisoning as belonging to the III series (see also Hoerburger and Fink, 1935) whilst Fischer and Duesberg (1932) have confirmed this finding in experimentally produced lead poisoning cases. Schreus (1935) and Hoerburger and Fink (1935), report that coproporphyrin III is present in the urine after the administration of salvarsan. Injury to the liver or interference with its excretory function, as in icterus, also leads to porphyrinuria, the pigment in this case proving to be coproporphyrin I so far as accurate investigations have been made. Watson (1935, b) identified coproporphyrin I in the urine of a case of cirrhosis of the liver occasioned by cinchophen. He (1935, c) also obtained the same pigment from the faeces in a case of familial jaundice at the haemolytic crisis and considers it to be most probably derived from the protoporphyrin of the red cells (cf. Van den Bergh, Grotepass and Revers, 1932).

As already stated, uroporphyrin has been isolated from the urines of several human cases of congenital porphyrinuria and in most instances the pigment was assigned to the I series. Very marked irregularities in the melting points of the octa-methyl esters have, however, occurred and this circumstance has led Fisher and others to suspect that mixtures of isomeric uroporphyrins might be present. Such isomers can hardly be position isomers since in some cases at least (Fischer and Duesberg, 1932) the uroporphyrin has been converted chemically into the corresponding coproporphyrin which proved to be identical with coproporphyrin I. A still further complication arises on account of the fact that two distinct types of porphyrinuria are recognisable, the chronic form (" Haematoporphyria congenita ") as typically exemplified in the case of Petry, and an acute form, " acute idiopathic porphyry " (Haematoporphyria acuta idiopathica), which presents certain characteristic features. Thus, acute idiopathic porphyry usually makes its appearance at a comparatively late stage in life, after adolescence; the attacks are periodic, and the excretion of porphyrin in the urine paroxysmal. Attacks are accompanied by severe colic, nervous symptoms and even muscular paralysis but pronounced photosensitivity, the most noticeable symptom in true congenital porphyrinuria, has never been recorded as an accompaniment to the acute form. Some tendency towards this condition is probably to be inferred, however, from the brown pigmentation of exposed skin surfaces and even blistering reported in the cases of Günther (1922), Brown and Williams (1909), and Eichler (1932).

These differences are well brought out in the review by Günther (1925) and the chemical aspect is now receiving attention from Waldenström, Fink and Hoerburger (1935). The melting points of the octa-methyl esters of the urine porphyrin found by different workers, both in cases of acute and of chronic porphyrinuria, may be summarised as follows. Cases of porphyrinuria due to poisoning by lead, trional, sulphonal, etc., are omitted.

Acute porphyrinuria.

Loeffler (1919)	262°	—
Weiss (1925)	274°	Faeces contained coproporphyrin I.
Fischer and Duesberg (1932)	269°	Decarboxylated to coproporphyrin I.
Waldenström, Fink, and Hoerburger (1935)	243°	Separated into fractions easily soluble in ethyl acetate (238°) and sparingly soluble (258°).

Chronic porphyrinuria.

Fischer (1915).....	293°.....	Petry case. Urine.
Fischer and Zerweck (1924, b).....	286–7°...	Petry case. Urine. Other fractions with lower M.P. prepared.
Fischer, Hilmer, Lindner, and Pützer (1925)	285°.....	Petry case. Bones.
Mackey and Garrod (1925–6).....	283°.....	Case G.L. Faeces corproporphyrin I (248°).
Jost (1927).....	273°.....	—
Fischer and Zerweck (1924, c).....	275°.....	Molzberger case. Raised only to 279° by saponification and re-esterification.
Van den Berg, Regniers, and Muller (1928) Van den Bergh, Muller, and Hijman (1929) Fischer, Platz, and Morgenroth(1929)	Copro, 160–1° Above specimen purified, 169°	Coproporphyrin III. Case known as "Van den Bergh's case".
Fischer and Duesberg (1932).....	Copro only 144–8° Remelt, 160–8°	Case K. Coproporphyrin III only.
Fischer and Duesberg (1932).....	281°.....	Case L.

It will be seen that only two cases of chronic porphyrinuria have so far occurred in which the pigment (coproporphyrin) excreted proved to belong to the III series. In all other instances the evidence suggests that the pigments belong to the I series.

Mention must be made of the case recorded recently by Van den Bergh and Grotepass (1933) of a man who for 20 years had suffered from a porphyrinaemia of gradually increasing intensity without, however, excreting more than the normal trace of porphyrin in his urine. Coproporphyrin I was isolated from the faeces but uroporphyrin could nowhere be found. The patient suffered from a fairly severe nephritis, his urine containing much albumin and one is led to suspect that the damage to the kidney was responsible for the failure to excrete an increased quantity of coproporphyrin. It might also be assumed, however, that the transformation of coproporphyrin to uroporphyrin normally took place in the kidney, a view for which there is some evidence (see Discussion later) and that in the diseased state of the organ in this particular case, such power had been lost. The site of formation of uroporphyrin is still not known with certainty. In true congenital porphyrinuria it would appear that some uroporphyrin, at least, arises in the bone marrow but in the acute form of the disease, this may not be the case. To the writer, it seems essential that acute and chronic porphyrinuria should be recognised as two different diseases, having a differing aetiology, and confusion avoided when speculating upon the course of the pigment metabolism in each.

Chemical Examination of the Swaziland Bovine Case.

The case was that of a young reddish-brown castrated male (2 years 4 months old). The animal was in poor condition and showed lesions round the eyes and muzzle resembling those caused by photosensitisation. In addition, in the centre of the back, just where the

hair fell away, there was an oval-shaped sore measuring 6 inches by 3 inches. The skin round this lesion was hard and keratinous (see Figs. 6 and 7). The appearance was consistent with the suggestion that the animal was photosensitive, but protected, over the greater part of its body, by the coarse reddish-brown hair of the coat.

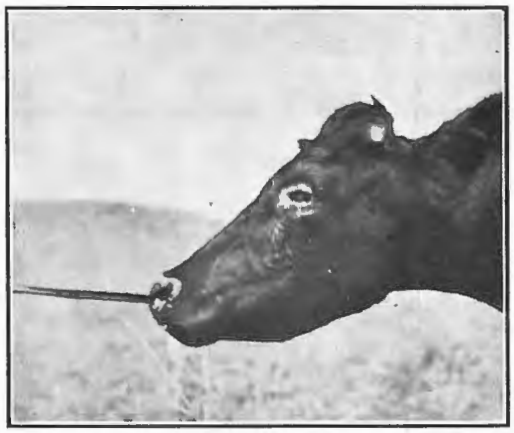


Fig. 6.—Bovine case of congenital porphyrinuria shownig lesions due to photosensitisation.

Fig. 7.—Same case after slaughter photographed to show keratinised epidermal sore on back.

Whilst under examination, it passed urine, a specimen of which was coloured a deep port-wine red colour and exhibited the following absorption bands (centres).* Fischer's (1916 b) figures for Petry's urine are given for comparison.

	614·0;	580-560;	540;	500.
Petry urine	614·5;	567·5;	535·5;	517.

* All spectroscopic measurements made with a Zeiss grating hand spectroscope. Centres of absorption bands quoted in $m\mu$.

The colour of the urine was seen to deepen on exposure to light.

The animal was anaesthetised by an injection of chloral hydrate and bled to death, the blood being collected with aseptic precautions in sterile evacuated flasks containing anticoagulant. In all, about five litres was obtained. The carcass was then cut open and the entire liver, spleen and kidneys removed as carefully as possible and wrapped in formalin-soaked muslin cloths. Specimens were collected in formalin and in alcohol for histological examination. The gall bladder was emptied into a sterile bottle, yielding about 300 c.c. of clear, dark-green bile. The entire skeleton was removed, cleaned from adhering muscle and wrapped in formalin cloths. It was noted that all calcified parts were deep chocolate brown in colour, whilst the cartilages and periosteum presented a normal appearance. A specimen of faeces was also collected from the large colon.

These materials were brought back to the Laboratory immediately and placed in cold storage. There was, in no instance, any indication of decomposition or putrefaction. The chemical examination was then conducted at leisure, taking one organ at a time and employing, as a general rule, the acetic acid-ether method introduced by Fischer followed by extraction of the residue with dilute ammonia to remove any uroporphyrin. Ammonia was found more convenient than pyridine and yielded excellent results.

Urine.

The bulk of the urine, which was free from albumin, was acidified with acetic acid and left in the ice-chest for several days until the precipitate had flocculated out and could be centrifuged off. The supernatant was still dark brown in colour but only exhibited a very faint porphyrin spectrum, together with a more marked broad band with centre 495·5 mμ. The precipitated porphyrin was esterified by methyl alcoholic hydrochloric acid, and the ester transfered to chloroform from which it was crystallised in the usual way. The crude uroporphyrin ester was washed repeatedly with boiling methyl alcohol, thereby eliminating a quantity of brown pigment possessing no absorption spectrum, and finally recrystallised several times. It had the usual appearance of uroporphyrin octa-methyl ester (see Fig. 8) and M.P. 275-7°.* The absorption spectrum in chloroform was as follows:—

626·1; 580·9; 570·8; 535·2; 500·7.
faint

Uroporphyrin methyl ester*:

626·1; 581·4; 570·5; 536·0; 500·8.

A specimen of the crude free porphyrin, dissolved in N/10 NaOH exhibited the following spectrum:—

614; 539·3; 502·8.

Uroporphyrin has 612; 539·0; 503·7.

It flocculated rather slowly on the addition of acetic acid.

* Melting points observed on the electrically-heated Kofler micro-melting point apparatus.

* Absorption spectra data taken from "Tabulae Biological", Vol. 3, or if not there recorded, from the original sources.

It was noticed that the mother liquors of the first one or two ester recrystallisations contained considerable quantities of pigment. When examined in a thick layer, the following absorption bands were seen to be present:—

642; 622·4; 602·2; 566·5; 531·8; 499·2.
faint

With the exception of the band at 642, these figures suggest a coproporphyrin. The solution was accordingly concentrated and left in the ice chest when a crystalline deposit formed consisting of a mixture of uroporphyrin ester and short stoutish prisms with slightly oblique ends. A partial separation was effected by repeated recrystallisation, the prisms melting ultimately at 233-5° and giving in chloroform the following spectrum, from which it was concluded that the substance was in all probability coproporphyrin I. (Compare however Fischer and Zerweck, 1924, b.)

623·6; 597·9; 567·3; 533·7; 498·6.

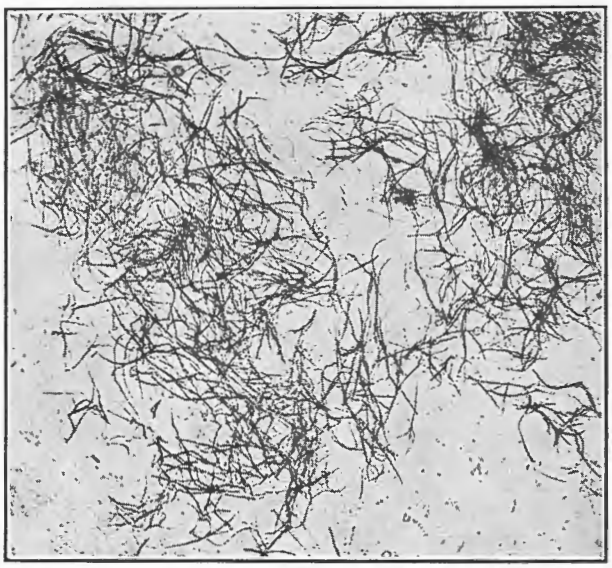


Fig. 8.—Uroporphyrin ester, M.P. 277°, from urine. × 245.

Coproporphyrin I was also isolated directly from a further quantity of the original urine by the acetic acid-ether technique. The ester in chloroform (M.P. 235°) had the spectrum:—

622·4; 596·5; 577·1; 566·8; 530·6; 497·7.

Coproporphyrin has

623·9; 597·3; 577·7; 568·2; 529·8; 497·9.

Transferred to 25 per cent. HCl it had:—

595·2; 575·4; 551·4.

Coproporphyrin has

593·9; 574·6; 550·9.

On shaking with chloroform, no pigment passed into the lower phase. Protoporphyrin was therefore absent.

Faeces.

The sample was extracted by the acetic acid-ether method and the pigment transferred to 2 per cent. hydrochloric acid. This solution was saken with chloroform, diluted ten times and again shaken, but no detectable quantity of deuteroporphyrin was extracted. The total porphyrin was therefore transferred to ether and from this to 10 per cent. sodium hydroxide solution. Only a very small precipitate of insoluble sodium salt separated during the course of the night. After filtration, the pigment was precipitated by neutralisation washed, dried and esterified. The ester crystallised in needle-like prisms M.P. 243-4° and exhibited the typical coproporphyrin spectrum

623·7; 597·7; 578·3; 568·6; 533·1; 497·3.

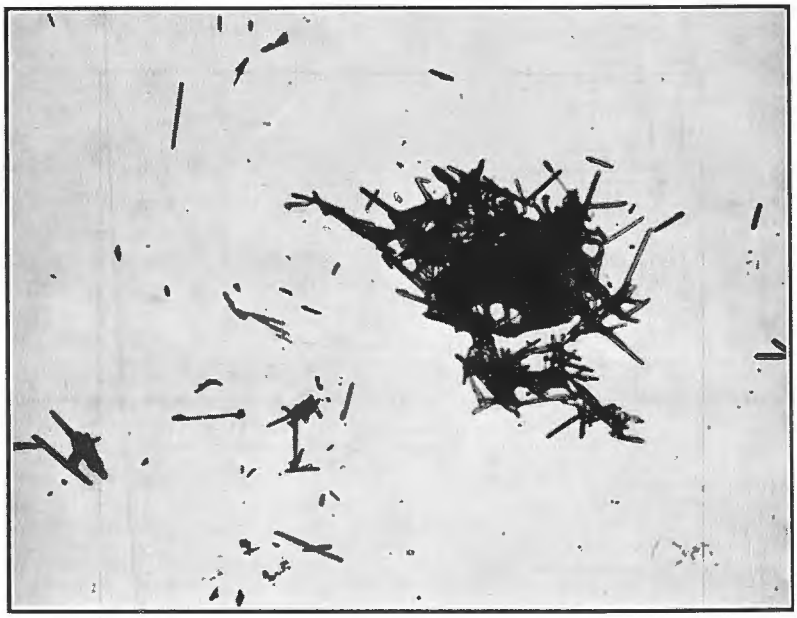


Fig. 9.—Coproporphyrin I ester, M.P. 243-4° from faeces. × 120.

It was therefore coproporphyrin I. (see Fig. 9). The mother liquor from the first crystallisation contained another much more soluble pigment with the following absorption spectrum with relative intensities of the bands as shown. It was not identified.

603·4; 574·9-551·3; 529·0; 499·0.

562·1 max.

III I II IV

As would be expected, there were present, in the original ether extract, porphyrins which did not pass into 2 per cent. HCl, most probably pigments derived from the breakdown of chlorophyll.

After removing these by shaking with 25 per cent. HCl, the ether was still rose coloured and exhibited the two-band spectrum of uroporphyrin copper complex:—

562·0; 526·5.

Cu uroporphyrin has 562·3; 528·2.

Blood.

The blood, 5 litres in all, was centrifuged and the red cell precipitate washed with isotonic saline. The plasma and cells were then worked up in the usual way.

(*a*) *Plasma.*

About 3·5 litres of yellowish plasma was obtained. It gave a very faintly positive direct Van den Bergh reaction.

The 5 per cent. hydrochloric acid shakings (about 1 litre in volume), had a purplish-blue colour with a strong reddish fluorescence. The absorption spectrum corresponded with that of a coproporphyrin. The residual ether contained no copper salt.

595·2; 575·4; 559·3-543·4. (551·4)

Coproporphyrin has 593·9; 574·6; 558·5-543·4. (550·9)

The pigment was transferred to ether and washed well. Spectrum in ether:—

625·4; (580·6); 568·8; 529·3; 507·6-485·2. (496·4)

Coproporphyrin has

623·9; 577·7; 568·2; 529·3; 505·6-490·3. (497·9)

On evaporation of the ether, a crystalline residue remained, stellate clusters of fine needle-like prisms. After washing with ether, the residue was esterified and the ester crystallised in slender needle-like prisms, M.P. 243-4° (see Fig. 10). The spectrum in chloroform confirmed the identification as coproporphyrin I.

623·0; (599·2); 577·5; 568·1; 532·4; 498·9.

Coproporphyrin ester has

622·3; 596·7; 578·1; 567·7; 532·9; 499·1.

The finding of coproporphyrin I in the blood plasma is of great importance since it offers an explanation of the photosensitisation from which the animal suffered, and brings the case into line with that of the man Petry and other chronic congenital porphyrinurics.

Fig. 10.—Coproporphyrin I ester, M.P. 243-4° from blood plasma. × 120.

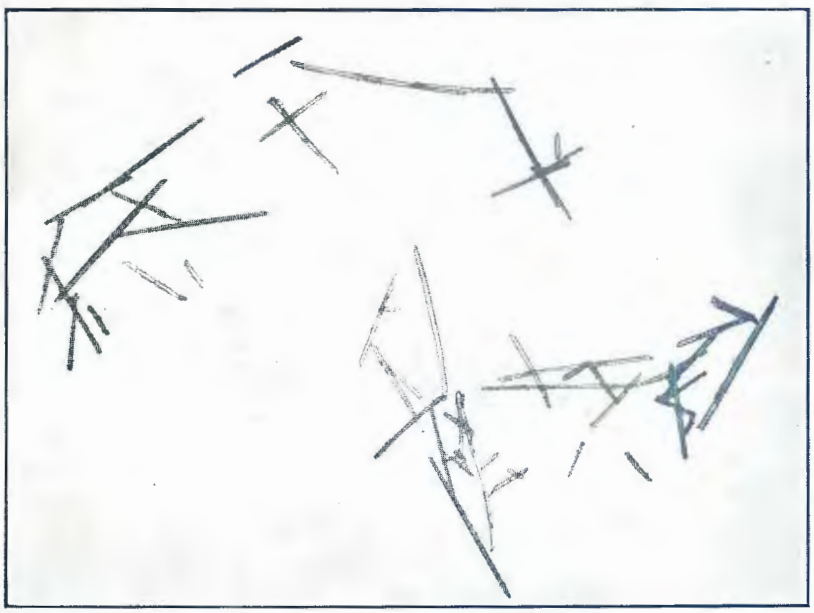


Fig. 11.—Coproporphyrin I ester, M.P. 241° from red blood cells. × 245.

The serum protein coagulum was extracted with pyridine with the intention of seeking uroporphyrin in the extract but by an unfortunate mischance, the pyridine extract exploded during concentration and was lost.

(*b*) *Red Cells.*

The final 5 per cent. acid extract from the erythrocytes had a greenish blue colour with a red fluorescence. On transference to ether, the following spectrum was exhibited:—

648; 624·2; 599·6; 582·5; 569·4; 530; 510.

Shaking with 25 per cent. HCl removed the porphyrins leaving some haematin behind in the ether.
The acid had

595·6; 579·5; 552·3.

On shaking with chloroform, only a trace of pigment (probably protoporphyrin) was removed. The acid spectrum now agreed fairly well with the acid spectrum of coproporphyrin:—

595·3; 576·3; 551·9.

Coproporphyrin has 593·9; 574·6; 550·9.

It was transferred to ether and washed well.

The ether solution had:—

623·6; 579·5; 567·9; 529·5; 498·0.
faint

Coproporphyrin has

623·9; 577·7; 568·2; 529·3; 497·9.

After removal of the solvent, the residue was esterified and recrystallised from chloroform. It formed slightly curved, needle-like prisms of M.P. 241° (see Fig. 11) and was therefore coproporphyrin I.

The spectrum in chloroform was as follows:—

622·3; 595·8; 577·8; 567·5; 531·4; 497·3.

Coproporphyrin ester has

622·3; 596·7; 578·1; 567·7; 532·9; 499·1.

The cell residue was shaken with dilute ammonia, the extract evaporated to dryness, esterified and the ester transferred to chloroform. A small quantity of pigment was obtained corresponding spectroscopically to uroporphyrin ester.

626·6; 570·0; 534·6; 501·1.

Uroporphyrin ester has

626·1; 570·5; 536·0; 500·8.

The identification of coproporphyrin I and of uroporphyrin in the erythrocytes is also of great significance as it suggests that some of the blood cells in circulation are definitely abnormal, containing the

Fig. 12.—Uroporphyrin ester from bones, 30 gm. lot, M.P. 273-4°. × 250.

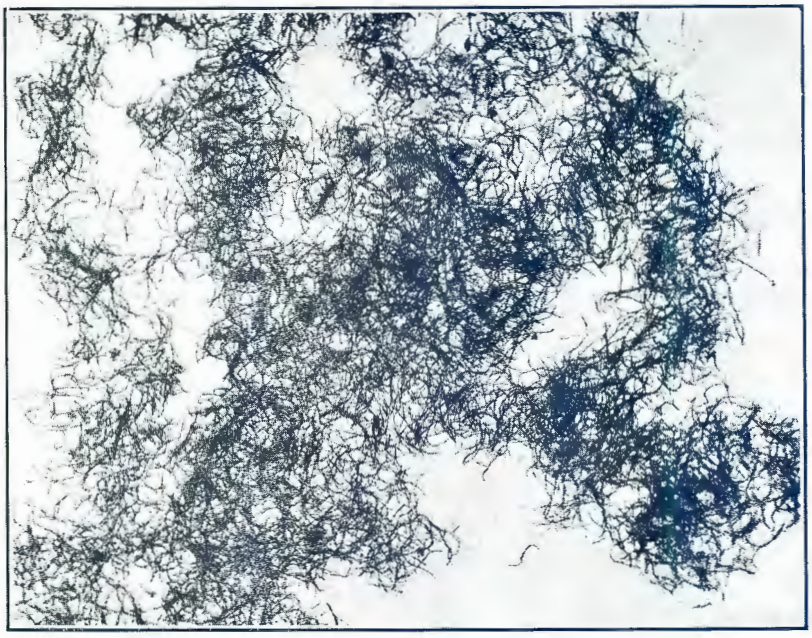


Fig. 13.—Uroporphyrin ester from bones, M.P. 276·7°. × 130.

atypical porphyrins produced in the bone marrow. Fischer did not apparently examine the cells of Petry's blood for porphyrins although he was able to show that the haematin was of the normal type.

Bones.

Material from two cases was available, the major part of the skeleton of the steer which was slaughtered and which has been described above and a small sample (30 gm.) of bone derived from a previous case. This latter material had been preserved in formalin. Upon it, various extraction methods were tried out and the most satisfactory technique, to be described below, employed when working up the larger sample. The limb bones, which were of a dark chocolate colour, exhibited in cross section a series of concentric rings of lighter and darker staining. The cartilages were uncoloured.

The material was crushed in a bone mill and defatted by continuous alcohol and ether extraction. The dry residue was then steeped in successive changes of 5 per cent. hydrochloric acid, the last traces of pigment being removed by 20 per cent. acid, but as this solution contained much dissolved bone substance it was worked up separately. The acid extracts were evaporated to dryness in a large vacuum evaporator and the crusty residue introduced into a large volume of methyl alcohol containing 5 per cent. by volume of concentrated sulphuric acid. Sufficient of this solution was used to ensure the complete precipitation of the calcium salts. After refluxing for 4 hours, the mixture was allowed to stand overnight at room temperature and then about one-tenth of the volume of chloroform added. Ice water was then poured in until the chloroform phase carrying the pigment separated sharply. Further shakings with fresh quantities of chloroform were added to the main solution. This was washed well with water, evaporated to dryness and the residue washed repeatedly with methyl alcohol until no more brown pigment, possessing no absorption spectrum, dissolved. The residue of uroporphyrin ester was then dissolved in a small volume of chloroform, filtered and crystallised by the addition of about 5 to 7 volumes of boiling methyl alcohol. The pigment separated in the finely microcrystalline form characteristic of uroporphyrin octamethyl ester, but the yield was very small. From the skeleton of the young steer, 0·3 gm. of pure pigment was obtained. The melting point was 273-4° in the first case and 276-7° for the material from the entire skeleton and could not be raised by repeated recrystallisation (see Figs. 12 and 13). The spectrum in chloroform corresponded to that of uroporphyrin ester:

626·0; 582·9; 571·3; 535·0; 501·2.

Uroporphyrin ester has

626·1; 581·4; 570·5; 534·7; 500·8.

A specimen of the crude extract of the bone had (in 20 per cent. HCl)

598·6; 578·1; 554·4; 463·9.

I II III IV Order III, IV, I, II.

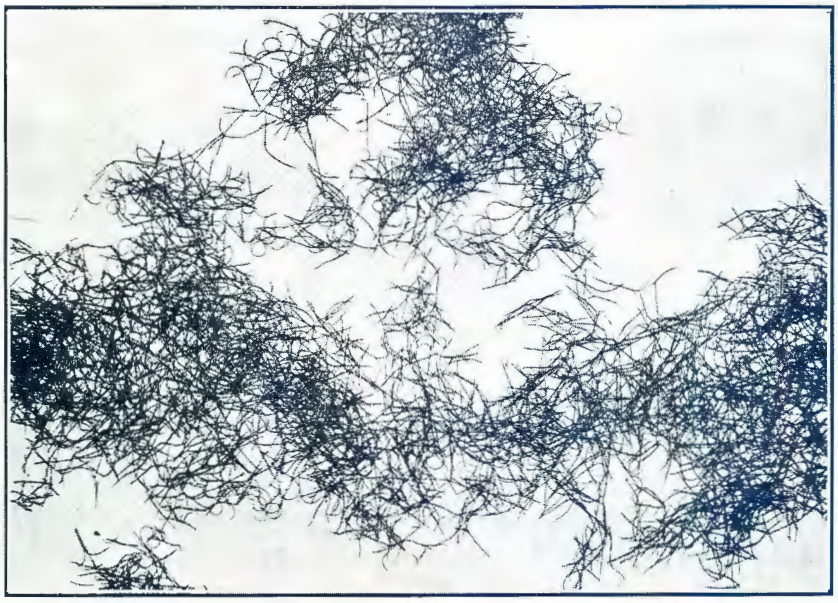


Fig. 14.—Uroporphyrin ester copper complex (Bones). × 120.

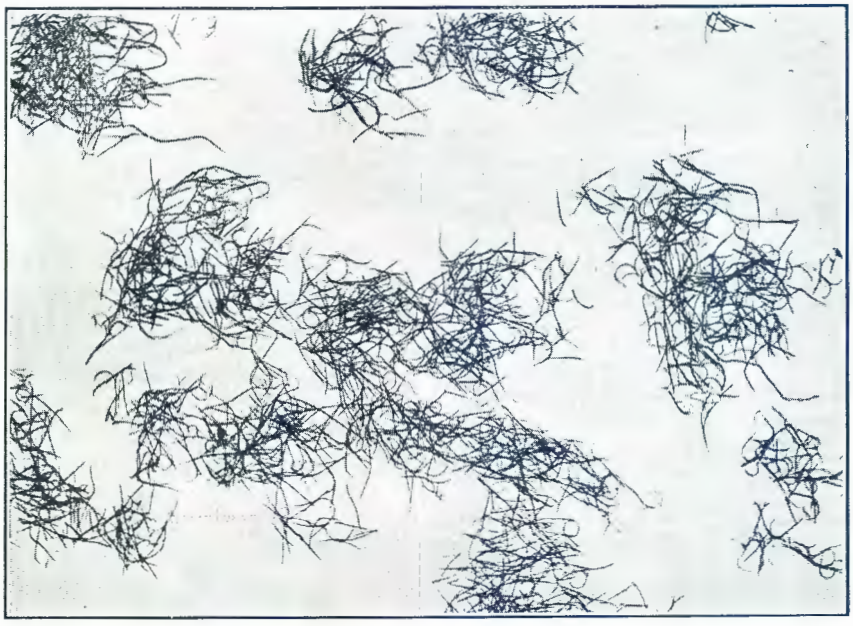


Fig. 15.—Uroporphyrin ester copper complex (Bones). × 120.

Uroporphyrin in 25 per cent. HCl has

597·9; 577·6; 553·6; 511·3.

The emission spectrum in ultra-violet light was measured using a small pocket spectroscope.

665-585 with bands at 625; 600.

Uroporphyrin has

664-596 with bands (660); 624; 602·5.

*Microanalysis**:

Uroporphyrin ester $C_{48}H_{54}O_{16}N_4$

	C	H	N	CH_3O
Found	60·73	5·94	6·20	25·43
Required	61·10	5·78	5·94	26·31

The copper complex prepared in the usual way crystallised from pyridine-acetic acid in fine, red, hair-like needles M.P. 311-4° and 310-3° respectively in the two cases (see Figs. 9 and 10). Fischer gives the M.P. of the copper complex of uroporphyrin from Petry's urin as 314° but it is to be noted that in many cases where the uroporphyrin sample had a M.P. of about 275° a copper complex with lower M.P. (about 292° has been obtained. The copper salts in the present instance were repeatedly recrystallised. The absorption spectra were measured in pyridine.

Preparation I ... ... 568·8; 532·6.
Preparation II ... ... 567·4; 532·5.

Uroporphyrin ester Cu complex has 570·0; 532·5.

It was noticed, when working up the larger preparation, that the mother liquors of the first two recrystallisations contained a considerable quantity of pigment. By adding ether until precipitation occurred and recrystallising repeatedly, a fraction crystallising in fine needles (see Fig. 16) was eventually obtained having M.P. 253-5° and the following spectrum in chloroform:

626·9; 572·0; 535·8; 500·9.

These figures agree well with uroporphyrin ester. The copper complex was also prepared (see Fig. 17) and found to have the normal melting point. The occurrence of a low-melting isomer in the bulk preparation is in accordance with the experience of others for urines, notably Waldenström, Fink and Hoerburger (1935), and probably accounts for the M.P. of the main sample being stationary but lower than that (293°) found by Fischer in the first instance for uroporphyrin from Petry's urine. The matter has already been discussed on a previous page. Coproporphyrin could not be detected in the bones.

* Microanalysis by Dr. O. Backeberg, University of the Witwatersrand, to whom my thanks are due.

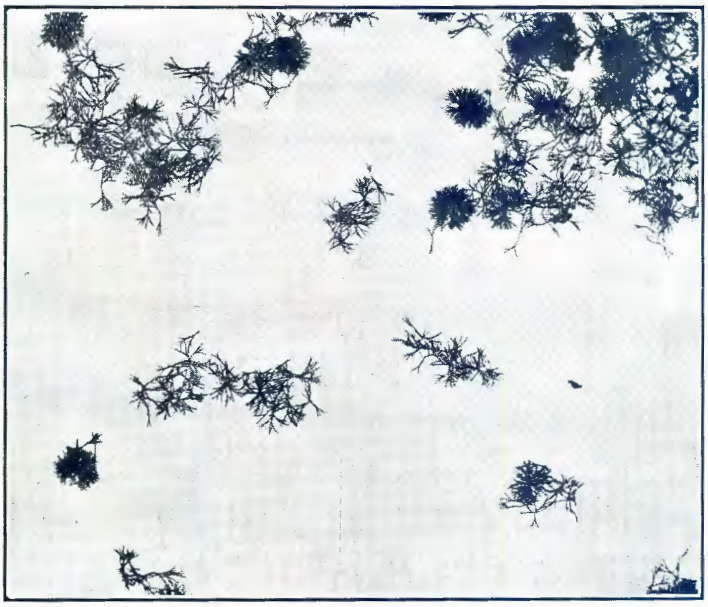


Fig. 16.—Uroporphyrin ester, M.P. 253-5°, from mother liquor of main crystallisation. × 120.

Fig. 17.—Uroporphyrin ester Copper complex from ester, M.P. 253-5°. × 120.

Bone Marrow.

This was obtained by longitudinal section of the long bones, the red marrow being removed. There was probably some admixture with pigmented bone lamellae. After defatting, the material was extracted with acetic acid-ether and this extract shaken with 5 per cent. hydrochloric acid, affording a strongly coloured acid layer exhibiting very clearly the following spectrum (the residual ether contained only haematin):

593·5; 550·9.

On transference to ether, a typical neutral coproporphyrin spectrum was obtained:—

623·0; 568·2; 528·8; 497·9.

Coproporphyrin has

623·9; 568·2; 529·8; 497·9.

The pigment was esterified and the ester, after recrystallisation, formed curved needle-like prisms M.P. 244-5° (see Fig. 18). It was therefore identified as coproporphyrin I.

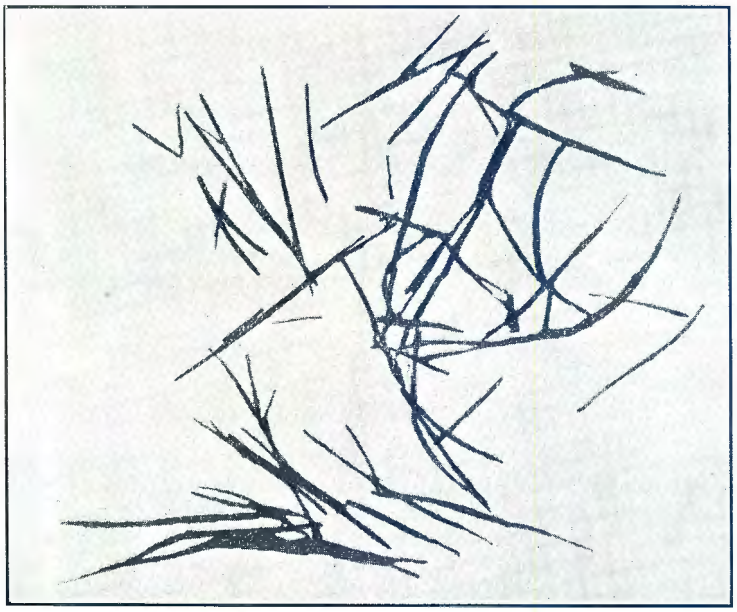


Fig. 18.—Coprophyrin I ester, M.P. 244-5°, from bone marrow. × 120.

The residue of material after extraction of the ether-soluble porphyrins was worked up for uroporphyrin in the way previously described. A good yield of ester was obtained (see Fig. 19) with M.P. 276-7° and spectrum

626·8; 581·7; 568·1; 534·2; 500·7.

The copper complex was also prepared and had in pyridine

565·0; 531·3.

The isolation of coproporphyrin I from the bone marrow and its absence from the bones is a highly significant finding, indicating as it does that the bone marrow is one, at least, of the sites where this particular pigment is synthesised

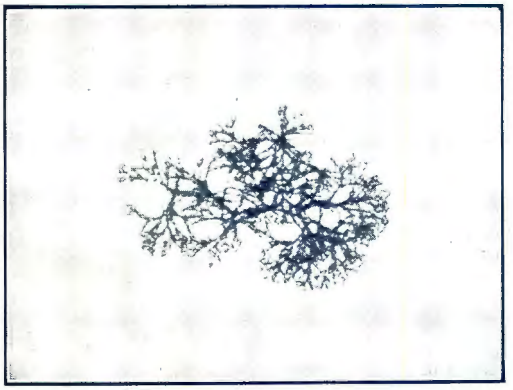


Fig. 19.—Uroporphyrin ester, M.P. 276-7°, from bone marrow. × 270.

Spleen.

The spleen was minced, steeped in glacial acetic acid and then worked up by the ether method in the usual way. The residue was subsequently extracted with a 5 volume per cent. solution of ammonia.

From the washed ethereal solution, 5 per cent. hydrochloric acid extracted a small quantity of ether-soluble porphyrin but insufficient to crystallise. The spectrum in ether corresponded to that of coproporphyrin.

624·0; 569·8; 529·5; 497·1.

Coproporphyrin has

623·9; 568·2; 529·8; 497·9.

The residual ether contained haematin. The ammoniacal extract exhibited bands at 576·5 and 541·8. It was evaporated to dryness and the residue esterified. The ester was washed with methyl alcohol and recrystallised from chloroform-methyl alcohol mixture. It had the appearance typical of uroporphyrin ester(see Fig. 20) and M.P. 278°, unchanged by repeated recrystallisation. The presence of these two porphyrins in the spleen is particularly noteworthy; uroporphyrin predominated.

Liver.

The minced organ was treated exactly as described above. The ethereal solution yielded to dilute acid only a very small quantity of a porphyrin corresponding spectroscopically with protoporphyrin. Coproporphyrin could not be found although the bile (see below) was found to contain large quantities of coproporphyrin. Most probably the correct explanation of this circumstance is that the excretory

function of the liver was in no way impaired and that coproporphyrin is normally eliminated together with other bile pigments via the biliary system [compare Van den Bergh, Grotepass and Revers (1932); Althausen (1931)]. Fischer and his collaborators (1925) in Petry's liver found coproporphyrin and uroporphyrin, together, possibly, with a trace of protoporphyrin. In fresh ox liver they were able to detect small quantities of protoporphyrin which was therefore regarded as a normal constituent.

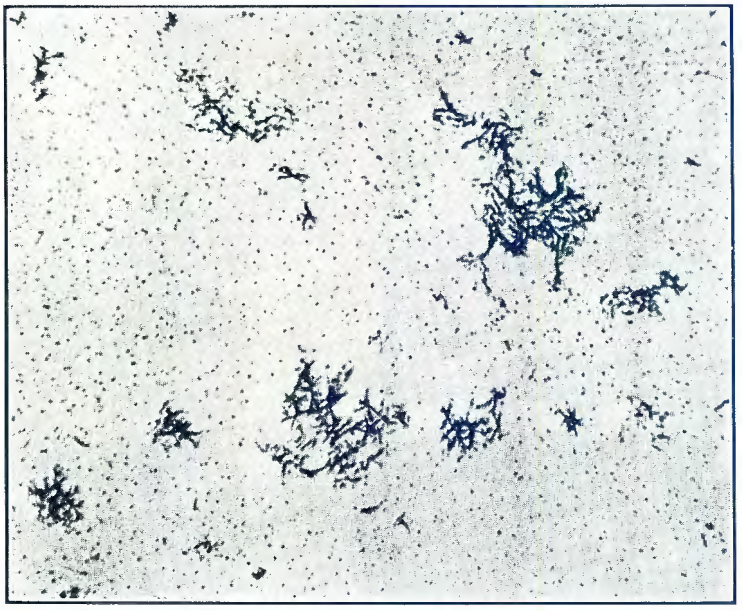


Fig. 20.—Uroporphyrin ester, M.P. 278°, from spleen. × 270.

The spectrum of the ether-soluble pigment from the present bovine case was as follows:—

(faint) 630·9; 575·6; 535·0; 500·1.

Protoporphyrin has

632·5; 575·8; 536·8; 501·9.

From the crude ester of the ammonia-soluble porphyrin, a considerable quantity of a dark brown accompanying impurity was removed by washing with methyl alcohol and the residue in chloroform showed an absorption spectrum indicating a mixture of uroporphyrin ester and its copper complex, thus:—

626·3; **566·0**; **530·0**; 501·1.

very intense

The entire material was therefore transformed into the copper salt which crystallised in fine, red needles, M.P. 313°, and exhibited in pyridine the characteristic two band spectrum:—

570·0; 532·8.

Uroporphyrin Cu salt has

570·0; 532·5.

Uroporphyrin and its copper complex would thus appear to be the only abnormal porphyrins present in the liver of this case.

Bile.

About 150 c.c. of the clear, dark green bile was used. This was mixed with acetic acid and extracted repeatedly with ether after which the residue was evaporated to dryness upon the water bath. The ether extract, after washing well with water, was shaken with 2 per cent. hydrochloric acid affording a deep purple-coloured lower phase. Ten per cent. acid, subsequently, removed only a small quantity of phylloerythrin. The 2 per cent. extract was shaken with chloroform until no more greenish-blue pigment came out (mesobiliviolin, etc.) and again shaken with fresh chloroform after dilution to 0·2 per cent. acid concentration. No mesoporphyrin or other pigment [compare Watson (1935, c) who isolated a previously undescribed porphyrin, ester M.P. 202-3°, from this fraction of the faeces in a case of familial haemolytic jaundice] left the aqueous phase. The entire porphyrin was therefore transferred to ether, affording a deep red solution with a very strong coproporphyrin absorption spectrum:—

623·9; 597·9; 577·4; 568·2; 529·3; 496·1.

Coproporphyrin has

623·9; 597·3; 577·7; 568·2; 529·3; 497·9.

Fig. 21.—Coproporphyrin ester from bile, M.P. 237°. × 225.

The ester was prepared (6·6 mgm.) and crystallised in the long curved needles characteristic of coprophyrin I (see Fig. 21). The M.P. was 237° and could not be raised by repeated recrystallisation. It was noticed that the mother liquors of the first crystallisation exhibited a faint band in the region of 645 indicating

the presence of small quantities of another pigment, probably protoporphyrin, which on standing in solution gives rise to such a band. Fischer et al. noticed a weak absorption band at 645·7 in the ether-soluble porphyrin fraction of Petry's bile.

No uroporphyrin could be detected in the evaporated bile residue after esterification, etc., by the usual methods.

Kidney.

The kidneys were somewhat dark in colour. They were minced and worked up exactly as described in the case of the liver and spleen. The 5 per cent. hydrochloric acid extract of the ether solution showed a well defined coproporphyrin spectrum but the quantity of pigment was too small to allow of crystallisation. Transferred to ether it had:—

623·9; 569·6; 529·0; 497·8.

Coproporphyrin has

623·9; 568·2; 529·3; 497·9.

Similarly, in the alkali-soluble fraction, uroporphyrin could be detected after esterification.

In chloroform it had:

628·1; 570·0; 535·5; 501·0.

Uroporphyrin ester has

626·1; 570·5; 534·7; 500·8.

In order to summarise clearly these results a chart is presented below indicating the pigments, with their ester melting points, isolated from the various organs. A similar chart showing the findings in Petry's case has been prepared, for comparison, from the published data of Fischer, Hilmer, Lindner and Pützer (1925). Where pigments were isolated in crystalline form and their identity confirmed by melting point determination, the entry is made in bold type, but where spectroscopic data alone was relied upon, italic type has been used.

Chart I.

Experimental Findings in Bovine Case of Congenital Porphyrinuria

Urine.....	Uroporphyrin I, 275–7°	Coproporphyrin I, 233–5°	Some urobilin and brown pigment without spectrum.
Faeces.....	*Uroporphyrin Cu Salt* (spectroscopically)	Coproporphyrin I, 243–4°	*Unidentified pigment* with absorption spectrum 603; 562; 529; 499.
Blood plasma.	—	Coproporphyrin I 243–4°	—
Blood cells....	*Uroporphyrin* (in traces, spectroscopically)	Coproporphyrin I 241°	—

CHART I—(*continued*).

Bones.....	Uroporphyrin I Case i 273–4° Case ii 276–7° (synthetic Cu salts 311-4° and 310–3° respectively)	Nil.	From mother liquors a more soluble uroporphyrin fraction 253–5°
Bone marrow.	Uroporphyrin I. 276–7	Coproporphyrin I. 244 5°	
Spleen.....	Uroporphyrin I. 278°	*Coproporphyrin* (spectroscopically)	
Liver.....	Uroporphyrin I and Cu complex 313°	Nil.	*Protoporphyrin* (spectroscopically; normal constituent). Much dark brown pigment spectroscopically negative.
Bile.....	Nil.	Coproporphyrin I. 237°, relatively large quantity	Possible traces of products derived from Protoporphyrin.
Kidney.....	*Uroporphyrin* (spectroscopically)	*Coproporphyrin* (spectroscopically)	- -

CHART II.

Experimental findings in human case of Congenital Porphyrinuria (Petry), compiled from data of Fischer et. al. (1925).

Urine.....	Uroporphyrin I. 293	Coproporphyrin I. 249 50°	— -
Faeces.....	Nil.	Coproporphyrin I. 250°	- -
Blood plasma.	Nil.	*Coproporphyrin* (spectroscopically; no M.P.)	
Blood cells....	—	- -	Haematin.
Bones.....	Uroporphyrin I. 280–3	Nil.	- -
Bone (scapula)	Uroporphyrin I. 285 *Cu complex* (spectroscopically)	- -	- -
Bone marrow..	*Uroporphyrin* (spectroscopically)	*Coproporphyrin* (spectroscopically)	—
Spleen.....	—	*Coproporphyrin* (spectroscopically)	—
Liver.....	*Uroporphyrin* (spectroscopically)	*Coproporphyrin* (spectroscopically)	*Protoporphyrin* (normal constituent, spectroscopically).
Bile.....	- -	Coproporphyrin I 247	—
Kidney.....	Uroporphyrin I 285	*Coproporphyrin* (spectroscopically)	
Heart.....	- -	*Coproporphyrin* (spectroscopically)	*Protoporphyrin* (spectroscopically).
Intestine.....	Nil.	*Coproporphyrin* and Cu complex (spectroscopically)	—
Pancreas.....	*proby. Uroporphyrin* (spectroscopically)	—	—
Muscle.....	—	*traces Coproporphyrin* (spectroscopically)	*traces Protoporphyrin* (spectroscopically).

DISCUSSION.

It will be seen from the accompanying charts that the investigation of this bovine case of congenital porphyrinuria has provided much additional information concerning the chemical nature and distribution of the pigments present in this disease. Thus, in the examination of the Petry material, the identity of the pigments (to which series they belonged, by melting point determinations) was achieved by Fischer and his collaborators (1925) in the case of the urine, faeces, bones, bile and kidney, these all proving to be series I pigments. As a result of the present investigation, however, the pigments have been identified by melting point as series I pigments in the following additional tissues; the blood cells and blood plasma, bone marrow, spleen, and liver. In general, the picture of the disease followed closely that seen in the man Petry.

The following four points, established among others, would seem to merit particular emphasis:—

(1) The bovine case of congenital porphyrinuria here studied exhibited clinical photosensitisation. It is thus brought into line with the chronic form of the disease in humans.

(2) Coproporphyrin I has been isolated from the blood plasma and erythrocytes, thus supplying a basis for explanation of the photosensitivity observed.

(3) The urine has been shown to contain uroporphyrin together with coproporphyrin I. No haemoglobin was present (compare Schenk, 1902).

(4) The bone marrow was rich in coproporphyrin I occurring with uroporphyrin. No protoporphyrin was detected.

Location of various Events in Pigment Metabolism and Modes of Excretion.

It is generally conceded that in the adult, haemoglobin is formed principally in the red bone marrow and that its transformation into bilirubin takes place in the cells of the reticulo-endothelial system, the liver playing an important part in this operation. As to the intermediate stages of both synthesis and degradation and the localities in which these changes take place, practically nothing is known. Thus, it is not proven that porphyrins form an obligate step in the normal synthesis of haemoglobin, although Borst and Königsdörfer (1929) are inclined to favour this view on account of their detection of protoporphyrin in the erythroblasts present in the red marrow, an observation in agreement with the findings of others. It has not been demonstrated, however, that the protoporphyrin here concerned belongs to the III series of isomers. However probable this might seem on general grounds, it can not be assumed as a basis for speculation, especially as a small quantity of protoporphyrin occurs normally in the erythrocytes and this would appear to belong, rather, to the I series.

The stages of the transformation of haemoglobin into bilirubin are just as uncertain. Iron must of course be eliminated at *some* stage but does this occur *before* or *after* the porphyrin ring is opened?

A reply to this question is possibly to be afforded by comparison with the system studied by Lemberg (1935) and would indicate an opening of the ring *prior* to the removal of iron, so that true porphyrins would at no stage be represented.

Warburg and Negelein (1930) had found that pyridine-haemochromogen in presence of hydrazine and molecular oxygen at 50° was converted into an iron-containing green pigment, exhibiting a characteristic absorption spectrum and which these authors regarded as a " green haemin ". Lemberg established the relationship of this substance to the bile pigments and was able to elucidate the course of the reaction. Pyridine haemochromogen (Fig. 22 No. I) is first

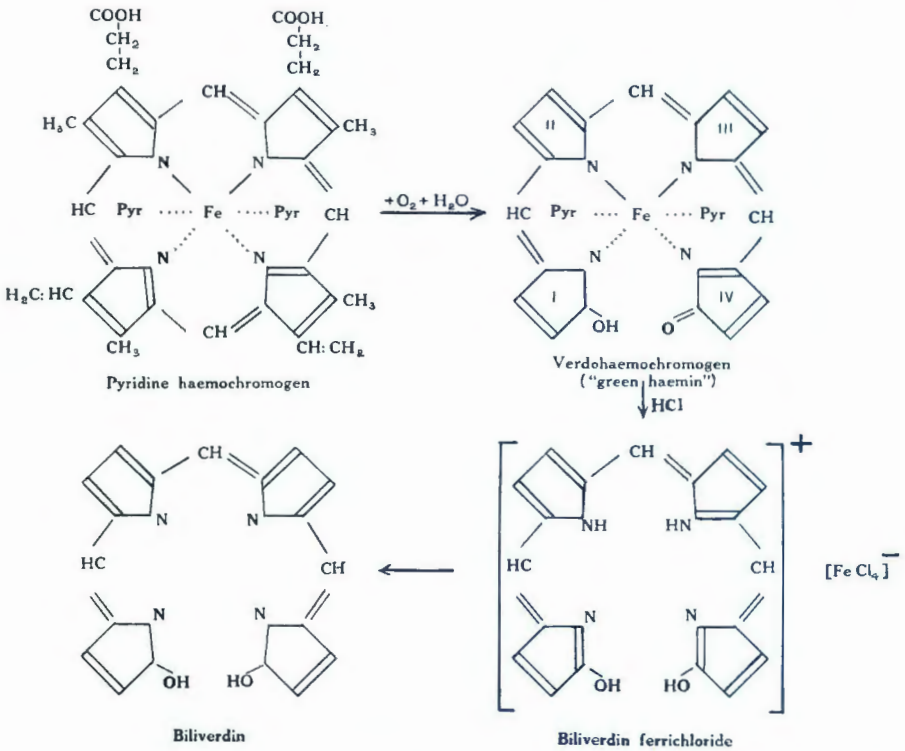


Fig. 22.—Scheme, after Lemberg, illustrating transformation of pyridine haemochromogen into biliverdin.

converted by oxidative rupture of the ring system into a verdohaemochromogen (No. II) which still retains the central part of the molecule intact. The scission occurs at the same point as in the physiological formation of bile pigment from haemoglobin and these verdohaems are to be regarded as iron salts of isobiliverdins. The latter are unstable and if set free, rearrange by migration of a hydrogen atom from the pyrrole nitrogen of ring III to the >CO group of ring IV. The action of hydrochloric acid upon verdohaemochromogen thus consists in the removal of pyridine and oxidation to verdohaemin.

This is then split into ferric chloride and *iso*biliverdin which rearranges to give biliverdin. Esterification of the carboxyl groups takes place simultaneously and the $FeCl_4$ salt of the ester crystallises out (No. III).

It is quite conceivable that a similar course of events may be followed during the conversion of haemoglobin, or globinhaemochromogen, into biliverdin in the living cell. The difference between bilirubin and biliverdin is, of course, only the state of the methine carbon atom, uniting pyrrole rings II and III, the grouping being $-CH_2-$ in the case of bilirubin. Lemberg is of opinion that biliverdin is most probably the first of the two pigments to arise in vivo. (Lemberg and Wyndham, 1936).

Haemoglobin breakdown, would not, under these circumstances, require the formation of porphyrin at any stage and it is significant as will be pointed out below, that in none of the simple haemolytic anaemias is a significant increase in porphyrin excretion ever observed.

With regard to the site of the formation of the abnormal pigments found in congenital porphyrinuria, it would seem, from an inspection of the present data, that there is fairly strong evidence to show that coproporphyrin I, and probably uroporphyrin also, arises in the bone marrow. The spleen may present a second locality of coproporphyrin synthesis. Such a distribution would be consistent with the viewpoint that coproporphyrin I arises as a by-product or anomaly during the attempted synthesis of haematin.

The conversion of coproporphyrin into uroporphyrin is a simple chemical matter merely involving the addition of four carboxyl groups. Uroporphyrin may thus arise secondarily from coproporphyrin and would appear to do so most probably in the bone marrow and spleen and possibly also in the kidney. That this latter organ is chiefly responsible for the change would be a tempting hypothesis. Uroporphyrin, being highly hydroxylated, is eminently suitable for urinary excretion just as coproporphyrin is more suited for excretion in the bile, and it is somewhat difficult to understand how so great quantities of uroporphyrin can be found in the urine when the circulating blood contains coproporphyrin but only traces of uroporphyrin. Rabbits' urine normally contains some uroporphyrin (Fischer and Zerweck, 1924, a; Stockvis, 1873; 1895).

The deposition of uroporphyrin in the bones is explicable on account of the very great affinity which calcified structures show for this porphyrin. Thus Fränkel (1924), was able to show that small amounts of uroporphyrin injected into normal growing guinea-pigs coloured the bones in exactly the same way as is seen in congenital porphyrinuria, whilst other porphyrins were ineffective or only effective when administered in large amounts. It is understandable, therefore, that small quantities of uroporphyrin existing in the blood stream would rapidly be taken up and fixed by the bony skeleton.

The bile contained no uroporphyrin but was rich in coproporphyrin whilst in the liver this latter pigment could not be detected. Since the liver was functionally sound (no icterus or marked increase in urinary urobilin) it is permissible to conclude that it may excrete

coproporphyrin from the blood stream with a high degree of efficiency into the bile. The urine also contained an amount of coproporphyrin I above the normal.

A transformation of coproporphyrin I into bile pigment would seem to be excluded on account of the isomerically different configurational relationship of the pigments in question and, moreover, the work of Lemberg discussed above renders improbable the intrusion of a porphyrin stage in the haemoglobin-biliverdin transformation. The bilirubin prepared by Fischer et. al. (1925) from Petry's bile was in no way abnormal.

Types of Anaemia and Porphyrin Excretion in various states.

Duesberg (1931) has critically compared the histological and chemical findings in different anaemic conditions, and as a result he is led to postulate the existence of at least two fundamentally distinct types of anaemia, that accompanied by increased regeneration processes and a type characterised by " impaired " or " disordered " regeneration.

Thus, whilst the appearance of the bone marrow was in all cases compatable with active erythropoetic efforts, there were in the former group of disorders signs of regenerative changes in the blood but no evidence of increased porphyrin excretion in spite of pronounced red cell destruction. Anaemias classed as belonging to the type with disordered regeneration were invariably accompanied by porphyrinuria. The following table will make this clear: —

Increased regeneration.	*Disordered regeneration.*
Increase in number of reticulocytes and increased oxygen consumption of blood. No porphyrin excretion.	Signs of regeneration in blood lacking. Porphyrin excretion.
Distilled water anaemia.	Lead poisoning.
Phenylhydrazine anaemia.	Sulphonal poisoning.
Haemolytic icterus.	Pernicious anaemia.
Saponin poisoning.	Congenital prophyrinuria.
Sodium nitrite poisoning.	
Anaemia by blood-letting.	

In the opinion of the writer, the differentiation should have been carried still further by a subdivision of the group in which porphyrin is excreted. Porphyrin elimination is only the objective end-result of a disturbance *at some point or other* of the normal pigment metabolism. The porphyrins are different in different types of intoxication. Firstly let it be again emphasised that simple haemolysis is unaccompanied by any significant degree of porphyrinuria even when the anaemia is intense as in phenylhydrazine poisoning, a fact which favours Lemberg's view of the direct formation of bile pigment from haematin, without passing over a porphyrin stage. All workers upon congenital porphyrinuria are agreed that the disturbance is not principally traceable to haemolysis.

Lead intoxication has, as a general rule, been regarded as leading to an anaemia of haemolytic origin, the excretion of a pigment derived from blood, latterly identified more exactly as coproporphyrin

belonging to the III series, being cited as evidence for such a pathogenesis. To the writer this appears erroneous. Haemolysis does not lead to porphyrin excretion and it seems far more likely that in lead poisoning there is an interference with the processes of synthesis, presumably a failure to complete the stage of *incorporation of iron* into the porphyrin complex and thus the pigment becomes physiologically useless, even dangerous, and is excreted [compare Naegeli (1931), " Das Blei ist, trotz der basophilen Punktierung der Erythrocyten, kein Blutgift, es ist ein Knochenmarksgift, das die Erythropoese schädigt."]. There is no marked excretion of uroporphyrin in lead poisoning, it will be noticed; the quantity of protoporphyrin in the erythrocytes is, however, increased (Van den Bergh, Grotepass, Revers, 1932).

Sulphonal intoxication presents a different picture. The anaemia is never so marked as in the case of lead poisoning and may even be absent (in experimental cases). The bone marrow is very hyperaemic and shows signs of erythropoetic activity, the number of erythroblasts, however, being again smaller than in lead poisoning. The porphyrin excreted in sulphonal intoxication is chiefly uroporphyrin I together with some coproporphyrin, i.e. pigments belonging to the abnormal or unphysiological isomeric series, not series III pigments as in lead poisoning, and it is significant that the anaemia is frequently rapidly compensated. There would, in this instance, appear to be an interference with pigment synthesis of such a nature that useless by-products are produced and excreted, *without, however, the normal line of chemical elaboration being interfered with.* A similar state of affairs is pictured by the writer as holding in chronic congenital porphyrinuria. Pernicious anaemia (excretion of coproporphyrin I in faeces but disappearance after liver therapy, Watson, 1935, a) would seem to possess several features in common with the above condition.

A Provisional Theory of Pigment Metabolism.

Whilst our meagre knowledge concerning the intermediate stages in blood synthesis and breakdown renders difficult any comprehensive theory of pigment metabolism, it is nevertheless possible and extremely helpful to construct schemes which illustrate the broad features of these processes. Such a scheme has been put forward by Whipple (1922) but this, of course, was not particularly concerned with the formation and excretion of porphyrins in diseases such as those under discussion. As a result of the present work upon congenital porphyrinuria and taking into account other pertinent data, a scheme has been evolved which, it is felt, is capable of affording explanations of the main facts observed.

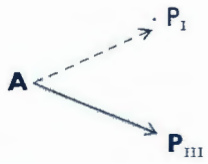
Thus, if it is assumed that coupling of substances containing pyrrolic groups and leading to the formation of porphyrins takes place in the erythropoetic tissues (bone marrow, etc.) as a normal step in the synthesis of haemoglobin, one can postulate that the possibility exists, on purely chemical grounds, of the elaboration in roughly equal quantities of four sets of isomers depending upon the relative positions of the substituent groups as in the four aetioporphyrins. Such would be the outcome of an in vitro chemical

synthesis. However, since the available raw materials (probably derivatives of proline or oxyproline) are themselves asymmetric and only one type of isomer is presented for the synthesis, it is conceivable that the posibilities might be limited by one half, that is to say, isomers belonging to only two series could be produced. Of the four aetioporphyrin series, only derivatives belonging to the I and III series are found in nature, and the former only in relatively small quantities.

Now, biological syntheses are almost invariably catalysed by enzymes or enzymic systems, themselves asymmetric, and producing an overwhelming preponderance of a certain optical or configurational isomer, together, perhaps, with traces of its enantiomorph. For example, the naturally occurring amino-acids are, with the exception of glycine, all optically active molecules and the enzymes concerned in their intermediary metabolism have very little if any action upon the non-physiological isomers. Similarly, a striking degree of specificity obtains among the enzymes acting upon the purine bases, position isomers belonging to a simple series. A counterpart in pure chemistry to this specificity of enzyme action is to be found in the decomposition of d- and l-camphocarboxylic acids in aqueous solution as catalysed by nicotine. Normally, in the absence of any catalyst, the rates of break up of the two isomers are identical:—

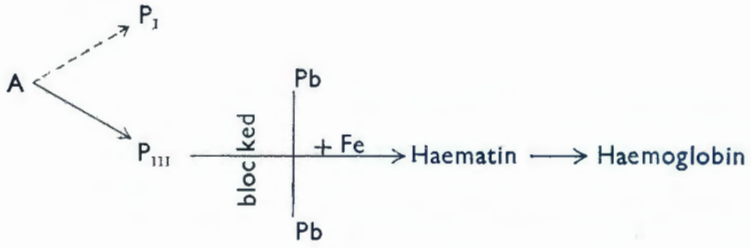
but when l-nicotine is added the relative rate of decomposition of the d-acid is markedly increased [Fajans (1910)].

One can therefore picture the events occurring in the bone marrow in the following way, where A represents the supply of asymmetric raw material and P_I and P_{III} the isometric porphyrin end-products produced. Since the change $A \longrightarrow P_{III}$ is selectively catalysed, it is shown in bold type whilst a dotted line indicates the production of small quantities of the by-product P_I

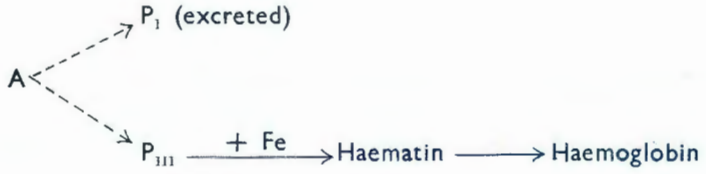


Protoporphyrin is probably the first formed and copro- and uroporphyrins arise from this as the result of further changes. The small quantities of series I porphyrins normally occurring in both animals and plants are thus to be regarded as quantitatively inferior and apparently useless by-products of the synthesis of the series III blood pigments.

Any interference with the elaboration of III series porphyrins into haematin would result in an anaemia accompanied by the excretion of coproporphyrin III but without any appreciable increase in the quantity of I series pigments normally present. This is precisely the state of affairs encountered in lead poisoning and it is understandable that compensation can only be effected, with the utmost difficulty, by an acceleration of the whole process of erythropoesis. Figuratively one may indicate the site of disturbance in lead poisoning thus: —



In congenital porphyrinuria (and possibly also pernicious anaemia), on the other hand, it would appear, as a reasonable explanation, that the disorder in pigment metabolism is due to a failure or inhibition of the selectively catalysed enzyme reaction $A \longrightarrow P_{III}$ thus resulting in the formation of P_I and P_{III} in quantitatively comparable amounts. As a result, to compensate for the threatened anaemia, and supply sufficient P_{III} for the requirements of haemoglobin formation, the whole level of porphyrin synthesis has to be raised and relatively large quantities of the useless series I pigments flood the organism and have to be eliminated. Congenital porphyrinuria may thus be analogous with alcaptonuria, cystinuria and other inherited errors of metabolism in that the organism is born lacking or deficient in a certain specific catalyst or enzyme necessary to complete a particular stage of intermediary metabolism. Sulphonal poisoning would appear to resemble congenital porphyrinuria in this respect that the selective action of the enzyme necessary for porphyrin III production is temporarily interfered with and an anaemia results, of no great intensity and easily compensated by an increased effort of activity on the part of the bone marrow but accompanied by a pronounced elimination of porphyrins belonging to series I.



The whole scheme of pigment metabolism in congenital porphyrinuria may thus be represented in the following way (see Fig. 23): —

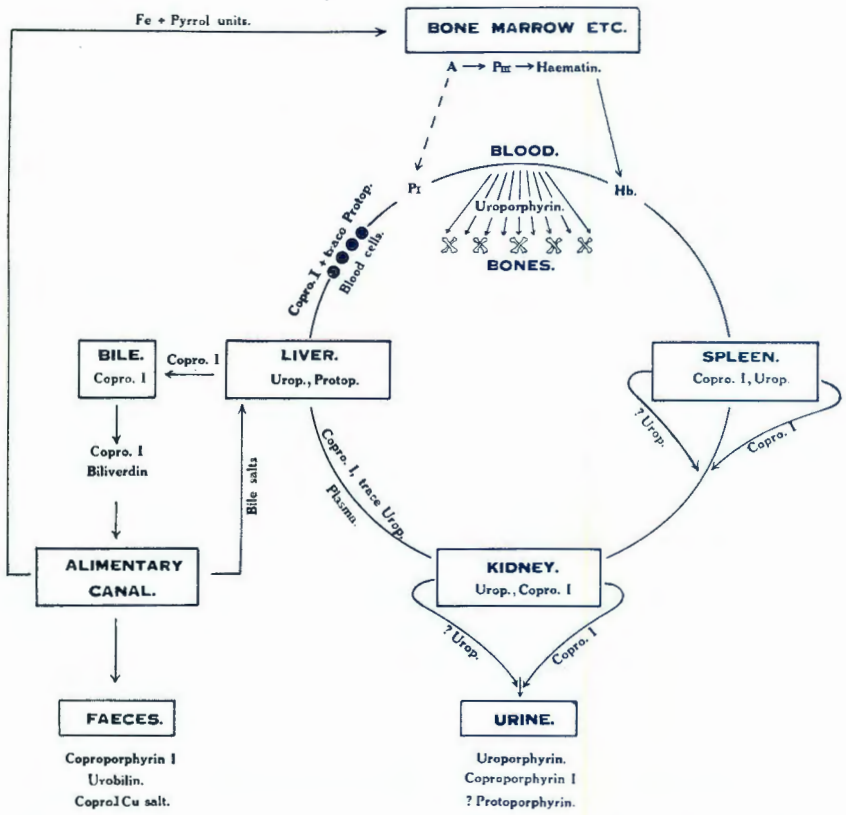


Fig. 23.—A provisional scheme of pigment metabolism with particular reference to the conditions obtaining in congenital porphyrinuria.

Incomplete and liable to error as the scheme may be, it should help, as a useful working hypothesis, to indicate further lines of investigation and by elaboration and improvement ultimately lead to a clearer understanding of the mechanisms underlying the synthesis of haemoglobin in the healthy subject and in various conditions of disease.

SUMMARY.

Of several living bovine cases of congenital porphyrinuria, discovered on a farm in Swaziland and all the progeny of a single pure-bred shorthorn bull (see Fourie, 1936), one animal was slaughtered for experimental purposes. This case, a castrated male, 2 years 4 months old, showed definite clinical symptoms of photosensitisation and passed a port wine-red coloured urine, exhibiting porphyrin absorption bands. The bones were found to be coloured a mahogany brown and on transverse section, concentric rings of lighter and deeper pigmentation were seen. The cartilages were normal.

Employing, in general, Fischer's methods, the individual organs and tissues were examined for porphyrins and pure crystalline materials (methyl esters) obtained as follows: Urine, *Uroporphyrin* (275-7°),*Coproporphyrin I* (233-5°); Faeces, *Coproporphyrin I* (243-4°) and its *Copper complex*; Blood plasma, *Coproporphyrin I* (243-4°); Erythrocytes, *Coproporphyrin I* (241°); Bones, *Uroporphyrin* (276-7°) and from a small sample derived from another case *Uroporphyrin* (273-4°); these esters had copper complexes 311-4° and 310-3° respectively. From the mother liquors of the main crystallisation was isolated a *Uroporphyrin* with ester M.P. 253-5° but yielding a normal copper salt. From Bone Marrow, *Uroporphyrin* (278°) and *Coproporphyrin I* (244-5°); Spleen, *Uroporphyrin* (278°); Liver, *Uroporphyrin* together with its *copper complex* (313°); Bile, *Coproporphyrin I* (237°). In other instances the yields of pure pigment were too small for identification by other than spectroscopic measurements (see chart in text of paper).

The significance of these pigments, belonging to the I series of porphyrins, is discussed in relation to normal haemoglobin synthesis and catabolism and the derangements of pigment metabolism occurring in disease and certain states of intoxication such as lead, sulphonal poisoning, etc. A suggestion is made as to the nature of the anomaly in congenital porphyrinuria and a provisional scheme of pigment metabolism mapped out.

I wish to thank Mr. G. Roets, B.Sc., for his generous assistance in the laborious task of working up the large quantities of material employed in this investigation and my colleague, Dr. Fourie, for the benefit of many discussions.

APPENDIX.

Examinations of Urine from Cases of Bilharziosis.

Since the disease bilharziosis is known to be accompanied frequently by the elimination of blood pigment in the urine and no reference could be found in the literature to any examination of such urines for porphyrin, it was deemed highly desirable to obtain firsthand evidence as to whether or not urinary porphyrin excretion is enhanced in this disease. The bovine cases of congenital porphyrinuria discovered in Swaziland were found on examination to be suffering also from bilharziosis. They have been treated for this condition and the infection apparently extinguished, nevertheless they continue to excrete large quantities of corporphyrin and uroporphyrin daily.

Through the kindness of Dr. F. G. Cawston of Durban, I was able to obtain about 250 c.c. of pigmented urine from an untreated human case of bilharziosis. A qualitative examination revealed the complete absence of uroporphyrin and the presence of traces of coproporphyrin in about the same quantity as is to be found in normal urine.

Some time later, Dr. A. Pijper of Pretoria was kind enough to procure for me 2·4 litres of urine pooled from three untreated human cases of bilharziosis then at the Pretoria General Hospital. This urine

had all been passed during the period December 18-19th and was received and examined at the Laboratory on the latter date (Dec. 19th).

In colour, the specimen was brownish with a slight mahogany tint; bilharzia ovae were present. It was acidified with glacial acetic acid to a final concentration of 5 per cent. and then shaken twice with 1·5 litres of ether. Some difficulty was experienced on account of emulsification. The ether extract was washed repeatedly with water and the porphyrin transferred to 5 per cent. hydrochloric acid affording a reddish solution exhibiting the acid porphyrin bands. After filtration through cotton wool, potassium acetate was added and the pigment again extracted with ether. This solution exhibited the following absorption bands: 623·4; (568·1); 529·1; 497·6.

The porphyrin was transferred again to 5 per cent. hydrochloric acid and the solution shaken with chloroform which removed some pigment including a trace of porphyrin, almost certainly protoporphyrin. The residual acid had a pale pink colour and showed the following bands, agreeing with those of coproporphyrin: 592·0; (572·2); 549·1.

It was compared with a standard porphyrin solution made by dissolving 1 mgm. of pure coproporphyrin I tetramethyl ester in 2 c.c. of concentrated hydrochloric acid and, when saponification was complete, diluting until the volume was 100 c.c. and the final acid concentration 5 per cent. 1 c.c. of the stock solution had to be diluted to exactly 1·5 c.c. for the intensity of the absorption bands to match that of the urinary porphyrin solution, the total volume of which was 20 c.c. Colour intensity comparison in a calorimeter afforded a similar result.

$\therefore$ 20 c.c. of urinary extract contained $20 \times \frac{1}{150}$ mgm.

$= 0 \cdot 133$ mgm. porphyrin.

This quantity of pigment was derived from 2,400 c.c. of urine.

$\therefore$ 100 c.c. urine contain $\frac{0 \cdot 133}{24}$ mgm.

$= 5 \cdot 56$ γ porphyrin.

($1\ \gamma = 1/1000$ mgm.)

Schreus and Carrié consider an excretion of 0 to 60 γ of coproporphyrin per day to represent the normal range, although figures as high as 80 γ were also obtained in some insetances. Günther considers 400 γ per litre to be pathognomic. Assuming an excretion of 1 litre per day by the patients investigated (it would certainly not be higher in this hot climate), it will be seen that the content of coproporphyrin found falls within the normal range.

Of the acid urine left after ether extraction, 750 c.c. was filtered through a column of active alumina and the chromatogram worked up by a method shortly to be described. Only a trace of pigment was obtained showing an absorption spectrum. It could not be shaken from ether solution to 5 per cent. hydrochloric acid and most probably represented a metal complex of coproporphyrin, since the following absorption bands were seen in pyridine solution. 564·0: 533.

For the sake of comparison with the above results, 320 c.c. of urine from a porphyrinuric bovine (No. 7018) was worked up quantitatively for coproporphyrin. The result was as follows:—

Volume of final solution in 5 per cent. hydrochloric acid = 500 c.c.

1 c.c. of this diluted to 1·1 c.c. matched 1 c.c. of standard porphyrin solution.

∴ total quantity of coproporphyrin present = 8·5 mgm.

or 1·72 mgm. per 100 c.c. of urine.

In concentration alone, this is 300 times the quantity found in the human bilharzia urine or in terms of daily excretion very considerably more (say approximately 1 to 2 thousand times as much). In addition, uroporphyrin was also present, of course, in considerable quantity in the bovine urine.

I wish to express my sincere thanks to both Dr. Cawston and Dr. Pijper for their kindness in placing the specimens at my disposal for examination.

Nature of the Uroporphyrin in the Bones from the Bovine Cases of Congenital Porphyrinuria.

As recorded in the accompanying article on page , the melting points of the uroporphyrin ester isolated from the bones and urines of those cases examined are lower than that recorded by Fischer for pure Uroporphyrin I methyl ester. In addition, a fraction was isolated from the mother liquors of the main crystallisation of the bone ester which appeared to be homogeneous and to have a greater solubility in methyl alcohol than the main product and also a melting point as low as 253-5°. The copper complex, prepared in the usual way, had melting point 300°.

Some months after these observations were made, the writer visited Europe and there learnt that Waldenström (1935, Deut. Archiv. Klin. Med. Vol. 178, pp. 38-49) and also Mertens (1936, Zeit. physiol. Chem. Vol. 238, p. 1) had independently and almost simultaneously succeeded in isolating Uroporphyrin III from urines in cases of *acute* porphyrinuria, the structure being proved by decarboxylation to coproporphyrin III. The melting point of Uroporphyrin III octamethyl ester was given as 255° to 258°; copper complex 304°. Uroporphyrin I was not present in these cases. I had with me a specimen of Uroporphyrin (M.P. 268°), very small in quantity (4·6 mgm.) but Dr. E. Mertens of the Eppendorfer Krankenhaus, Hamburg, very kindly offered to carry out the decarboxylation of this sample according to the technique previously used and to compare the copper complex of the coproporphyrin ester with that previously obtained by her from Uroporphyrin III. The experiment yielded only the derivative of the series I ester and by the removal of the copper and crystallisation, the identity of the porphyrin (ester M.P. 245°) with coproporphyrin I was further confirmed. Such a result indicated that the original uroporphyrin sample of low melting point was an impure uroporphyrin I or possibly uroporphyrin I in loose combination with some accompanying material. Compare

Fischer and Duesberg (1932) whose preparation of M.P. 269° also only yielded coproporphyrin I on debarboxylation. Shortly afterwards, Fischer and Libowitzky (1936; Zeit, physiiol. Chem. Vol. 241, pp. 220-2, Nachschrift bei der Korrektur) claimed to have separated a low melting point uroporphyrin ester derived from Petry (? bone), M.P. 286°, by means of adsorption analysis into a uro-III ester of M.P. 261° and a uro-I ester of M.P. 302° (uncorr., 311° corr.) The technique is not described, neither was the identity of the uroporphyrin III ester proved by degradation to the corresponding coproporphyrin.

The results of Fischer and his co-workers would thus seem to conflict with the present writer in whose low melting point preparation, as stated, Dr. Mertens found only uroporphyrin I unless uroporphyrin III was also present and coproporphyrin III being so much more difficultly crystallisable than the I isomer had not been detected in the products of decarboxylation. It must be admitted, however, that the specimens in the present instance had not been purified by chromatographic adsorption.

I wish to express my sincere thanks to Dr. E Mertens for her very friendly and valuable collaboration and for permission to make use of the results she obtained in this way. My thanks are also due to Professor O. Schumm, the Principal of the Institute where the experiments were actually carried out.

Chromatographic separation of the Uroporphyrin into two Isomers.

Attempts to separate the unesterified porphyrin chromatographically having given somewhat disappointing results, (these experiments will be recorded later) attention was directed to the treatment of the esters by the chromatographic method. In the search for a suitable solvent, the observation was made that uroporphyrin octamethyl ester is easily soluble in hot dioxan (diethylene dioxide) but separates from this solvent on cooling in fine crystalline form. It is also possible to effect a high degree of purification by two or three repeated recrystallisations from this solvent since the low melting isomer is apparently much more soluble and remains largely in the mother liquors. Thus, a specimen of uroporphyrin ester from bovine urine which had from chloroform-methyl alcohol a melting point practically constant at 278° was found to melt after one crystallisation from dioxan at 289-290° and after a second recrystallisation at 291-293°.

Dioxan is a solvent eminently suited to the chromatographic method; alumina (Merck's ' nach Brockmann ') was found to be a better adsorbent than either calcium carbonate or talc. The above specimen when put through the column diffused fairly rapidly and homogeneously downwards being washed out by fresh dioxan and leaving a small mahogany coloured ring near the top of the column. The mother liquors of the first recrystallisation, similarly treated, gave a homogeneous rapidly diffusible fraction, crystallised by addition of hot methyl alcohol to the dioxan solution, M.P. 292-293°, and a narrow ring of pigment which was eluted by pyridine or by

chloroform containing acetic acid (methyl alcohol containing 5 per cent. by volume of sulphuric acid is a still better eluting agent) and crystallised in the usual way. It separated in the typical uroporphyrin form, had M.P. 260° and gave the following absorption bands in chloroform:

625·8; 572·1 *maximum;* 535·0; 500·0.

This material would appear to be the ester of Uroporphyrin III. When similarly treated, the uroporphyrin, separated from the bones of the bovine case reported upon in this paper, (M.P. 276-7°) was resolved into pure uroporphyrin I the melting point of which could not be raised above 293° by repeated adsortion, and a small quantity of the other isomer M.P. 261° which it would appear is uroporphyrin III. The discrepancy between the various melting points recorded in the literature would thus be explicable upon the basis that small amounts of uroporphyrin III accompany the I series porphyrin in the congenital form of the disease.

The coproporphyrin ester which had been obtained from the porphyrinuric bile (M.P. 237°) was dissolved by warming in a little dioxan and filtered through an alumina column. The chromatogram on development with dioxan afforded a sharply defined, narrow ring near the top and a more rapidly diffusing fraction. This latter was coproporphyrin I (M.P. 246-8°) whilst the small amount of pigment retained more tenaciously appeared also to be a coproporhyrin.

Spectrum in chloroform: 626·2; 563·5 *maxmum;* 529·5; 496·7.

Further work in this direction is in progress.

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