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Isotopic Studies of the Conversion of Oxophlorins and their Ferrihaems into Bile Pigments in the Rat

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1. Tritiated oxymesoporphyrins and their ferrihaems were tested as possible intermediates in the catabolism of haemoglobin. The tritiated compounds were injected into rats with biliary fistulae and the incorporation of the isotope into bile, bile pigment, urine, faeces, liver, kidney and spleen was measured. 2. α-Oxymesoferrihaem was extensively converted into bile pigment and specifically to the expected mesobilirubin. 3. β-Oxymesoferrihaem was poorly converted into bile pigment and was not converted into mesobiliverdin IXβ. The latter was independently shown to be excreted rapidly in bile. 4. The free oxyporphyrins were also poor precursors of bile pigment, and α-oxymesoporphyrin competed with bilirubin for excretion by the liver. 5. By analogy with the results obtained with α-oxymesoferrihaem it is concluded that α-oxyprotoferrihaem is an intermediate in the catabolism of haemoglobin, undergoing further oxidation to bile pigment under the catalysis of an enzyme of definite specificity.

The mechanism of the conversion of erythrocyte haemoglobin into bile pigment is obscure. The earliest theory was that oxidative fission of the protoporphyrin ring occurred after the separation of iron and globin, leading directly to biliverdin, which was then reduced to bilirubin in the cells of the reticulo-endothelial system (for review see Lemberg & Legge, 1949). Lemberg (1956) thought that haem with a carbonyl group replacing one methene bridge and still bound to globin was a possible intermediate. Such a compound may be converted directly into bile pigment by removal of globin and iron, loss of carbon monoxide and further oxidation, or may give rise to verdohaems still containing an intact macro ring of only 19 carbon atoms and a single oxygen bridge. There is now evidence that the reaction is enzymic in nature. Nakajima (1963) and Nakajima, Takemura, Nakajima & Yamaoka (1963) claimed that a haem methenyl oxygenase converted certain haem derivatives into a formylbiliverdin, which was converted further into a biliverdin. Tenhunen, Marver & Schmid (1969) have since isolated a microsomal oxygenase that specifically catalyses the α-fission of haem with production of biliverdin. On the other hand Ó Carra & Colleran (1969) have marshalled important evidence that the catabolism of haem in haemoproteins is catalysed by the protein moiety itself. This is envisaged as providing, within the haem-accommodating crevices, a hydrophobic environment for the α-methene bridge, ensuring fission at that site. Such specificity, maximal for myoglobin, which gave only IXα-pigment on incubation with ascorbate, was only partial for haemoglobin, which afforded up to 35% of its haem as IXβ-verdin. Ó Carra & Colleran (1969) also found enzymic reduction of the IXβ-verdin to rubin to be much slower than for the IXα-isomer, and attributed its absence from natural bile pigment (Gray, Nicholson & Nicolaus, 1958) to degradation and elimination by other routes.

The structures of the tetrapyrrolic compounds intermediate between haem and bile pigments have not been established with certainty, and studies of haem degradation *in vitro* have been hindered by the lack of pure isotopically labelled intermediates. However, tritiated oxyporphyrins (oxophlorins) with pyrrole β-substituents similar to and in the same order as those in the prosthetic groups of

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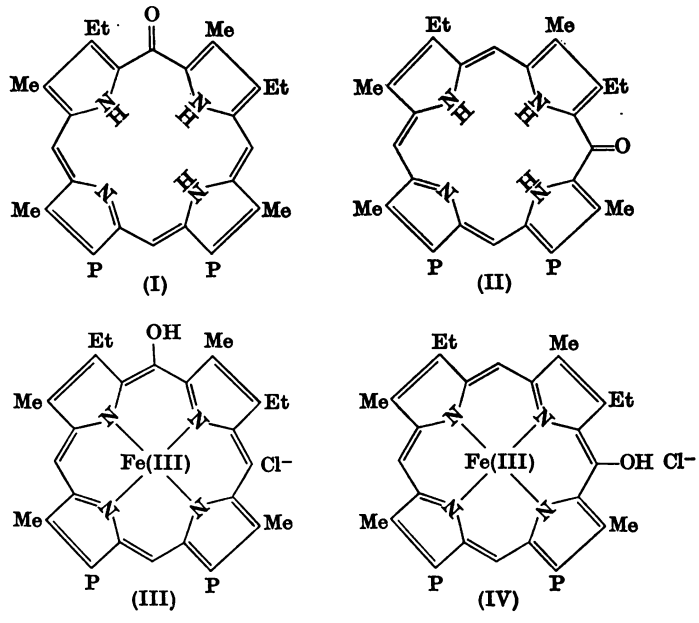


Me = CH_3
Et = CH_2CH_3
P = $CH_2CH_2CO_2H$

natural haems have been synthesized by Jackson, Kenner, McGillivray & Smith (1968*a*) and Crook, Jackson & Kenner (1971). These compounds have one methene bridge of the corresponding porphyrin replaced by a carbonyl function, which can readily isomerize to the hydroxymethene form to allow chelation of metal in haem formation (Jackson, Kenner & Smith, 1968*b*). α-Oxymesoporphyrin (I), β-oxymesoporphyrin (II) and the chlorides of the corresponding ferrihaems (III) and (IV) have been obtained with bridge oxygen functions in the α- or β-positions and with tritium in the γ- or δ-positions respectively. The corresponding divinyl compounds, more closely related to protohaem, have not yet been synthesized. The present paper describes studies of the metabolism of the meso compounds in rats with external biliary fistulae.

METHODS

Biological methods

Animals. Wistar rats weighing 380–420g from the King's College Hospital Medical School colony, fed the M.R.C. no. 41 diet, were anaesthetized with ether and at laparotomy a Portex 00-gauge flexible polythene tube was inserted into the bile duct as described by Ostrow, Hammaker & Schmid (1961). The animals were then transferred to restraining cages and provided with unlimited water. Bile was collected in a 20ml test-tube at 0°C with as complete protection as possible from light. To avoid possible coupled oxidation of ascorbic acid and haem, ascorbic acid was not added.

Preparation of solutions of tetrapyrroles for injection. The tetrapyrroles, the quantities used and their radioactivities are summarized in Table 1. Each tetrapyrrole was dissolved in 0.1ml of 0.1M-NaOH and added to 1ml of rat plasma. Then 0.1ml of 0.1M-HCl was added and the solutions were injected into the tail veins of rats with external biliary fistulae.

Treatment of bile. Bile was collected over four 2h periods and for a further 16h overnight. Separate 0.1ml quantities from each collection were used for measurement of total radioactivity and for the determination of bilirubin by the method of Malloy & Evelyn (1937). The remainder of the bile was used for the isolation of a fraction that would contain bilirubin and mesobilirubin.

Chemical methods

Preparation of tritiated oxymesoporphyrins and their ferrihaems. α- and β-Oxyporphyrin were synthesized by the methods of Jackson *et al.* (1968*a*,*b*) and were tritiated by exchange in tritiated acetic acid. This exchange occurs preferentially at the methene bridge opposite that which bears the oxygen atom (Jackson *et al.* 1968*a*). The corresponding ferrihaems were obtained from the oxyporphyrins by treatment with $FeSO_4$ as described in the following preparations, which were performed by Dr R. P. Carr and Dr P. Crook.

Table 1. *Specific radioactivities of tritiated tetrapyrroles and the quantities injected into rats with external biliary fistulae*

Compound	Sp. radioactivity (μCi/mg)	Radioactivity administered (μCi)
α-Oxymesoferrihaem	0.07	(1) 0.07
		(2) 0.07
		(3) 0.04
β-Oxymesoferrihaem	0.20	(1) 0.27
		(2) 0.12
		(3) 0.16
		(4) 0.04
α-Oxymesoporphyrin	0.02	0.08
β-Oxymesoporphyrin	0.07	0.003
Mesobiliverdin IXβ	0.01	0.001

Tritiated α-oxymesohaemin IX was prepared from α-oxymesoporphyrin IX dimethyl ester (50mg) by solution in a mixture of acetic acid (0.12ml) and chloroform (0.4ml) containing tritiated water (20mCi). After 43h in the dark active solvent was removed under reduced pressure and residual oxophlorin was dissolved in acetic acid (4ml) containing sodium acetate (5g), NaCl (1.0g) and $FeSO_4$ (0.4g). The mixture, after being heated on the steam bath (15h), was diluted with water and chloroferrihaem ester was extracted into methylene chloride. The solution was dried (over $MgSO_4$) and solvent was removed. The solid was stored in the dark (16h) in tetrahydrofuran (20ml)–conc. HCl (2ml) mixture, t.l.c. then showing complete hydrolysis of the ester. The crude chloroferrihaem diacid (33mg) was obtained by evaporation of the solution to dryness, washing with water, drying and trituration with light petroleum (b.p. 60–80°C). The β-oxymesoferrihaem was similarly prepared from β-oxymesophorphyrin dimethyl ester.

Isolation of bilirubin and mesobilirubin from bile. A fraction that contained bilirubin and mesobilirubin was isolated from the bile by the method of Ostrow *et al.* (1961) with minor modifications. Pigment glucuronides were precipitated by the addition of 10% (w/v) lead acetate solution and after centrifugation the precipitate was hydrolysed for 0.5h in 5ml of cold M-NaOH containing 15mg of ascorbic acid. After neutralization with M-acetic acid pigments were extracted into chloroform and the chloroform solutions were washed successively with 1% ascorbic acid solution, 10% (w/v) NaCl, 10% (w/v) ascorbic acid and finally water. The chloroform was dried by filtration and evaporated at 40°C and the pigments were crystallized at least twice from chloroform–methanol.

Partial separation of bilirubin and mesobilirubin by t.l.c. The method of Petryka & Watson (1968), with E. Merck A.-G. (Darmstadt, Germany) polyamide adsorbent, was used. Plates were prepared by using 10g of adsorbent in 45ml of methanol applied to the plates by using a Uniplan leveller and a spreader (both from Shandon Scientific Co. Ltd.), and were dried at room temperature for 2h before use. A sample (50–100μg) of the crystalline pigment in 0.08–0.1ml of chloroform was applied to each plate by using the Camag Chromatocharger.

Ascending chromatograms were developed over a distance of 18cm at room temperature with methanolic aqueous ammonia [methanol–aq. 10% (w/v) ammonia–water (9:1:2, by vol)]. Mesobilirubin (R_F 0.67) moved just ahead of the bilirubin (R_F 0.56), but the two pigments were not completely separated.

After the chromatograms had been dried successive fractions were removed by suction of the adsorbent into a small glass bulb containing a sintered-glass disc. Pigment from each fraction was eluted by minimal quantities of 10% (w/v) ammonia in methanol and the extracts were transferred to vials for measurement of radioactivity.

Measurement of radioactivity. After evaporation of the solvent, the solid material was dissolved in 14ml of dioxan phosphor prepared by dissolving naphthalene (60g), 2,5-diphenyloxazole (PPO) (4g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) (0.2g) in a mixture of dioxan (900ml), toluene (100ml) and ethanol (50ml). The extinction of each fraction was measured at 450nm and the bilirubin concentration was calculated from millimolar extinction coefficient of bilirubin in dioxan phosphor, which was previously determined as 38.2 for bilirubin (of millimolecular extinction coefficient 66 at 450nm in chloroform).

A sample (50–100μg) of crystalline pigment or 0.1ml each of tissue homogenate or faecal homogenate was added to 1ml of methanolic 0.5M-Hyamine. Similar solutions of 0.5–1.0ml of urine, or the whole specimen where the volume was less than 0.5ml, were made with 1ml of methanolic 0.5M-Hyamine and all the preparations were diluted to 15ml with dioxan phosphor. The radioactivity was measured in the Packard Tri-Carb model 3380 liquid-scintillation spectrometer. The radioactivities of bile solutions and tissue suspensions were counted with average efficiencies of 24 and 12% respectively.

RESULTS

Distribution of tritium from α- and β-oxymesoferrihaem. In two experiments with α-oxymesoferrihaem about 30% of administered radioactivity was excreted in the bile within 24h, another 30% in the urine and the remainder was present in almost equal amounts in the liver, kidney and spleen (Table 2). The cumulative excretion of radioactivity during 24h is shown in Fig. 1. The measured specific radioactivity of the extracted pigments and the amount present in the bile showed that at least 95% of the

Table 2. *Mean percentage distribution of radioactivity into bile, bilirubin, urine, faeces, liver, kidney and spleen during* 24h *after the administration of tritiated α-oxymesoferrihaem, β-oxymesoferrihaem and mesobiliverdin IXβ to rats*

The quantities of tetrapyrroles used are given in Table 1.

	Distribution of radioactivity (%)		
Tetrapyrrole injected ...	α-Oxymesoferrihaem	β-Oxymesoferrihaem	Mesobiliverdin IXβ
Bile	30*	4.2†	64†
Bilirubin	27*	4.0†	—
Urine	32*	3.8†	6.3†
Faeces	4†	0	3.1†
Liver	19†	18*	20†
Kidney	11†	0.66*	8.5†
Spleen	19†	0.55*	7.6†

* Mean of three experiments.
† Mean of two experiments.

radioactivity of the latter was in the fraction containing bilirubin and mesobilirubin.

With β-oxymesoferrihaem the incorporation of radioactivity into the bile in 24h was only about 4% in three experiments. This radioactivity was almost wholly accounted for by the radioactivity of the bilirubin–mesobilirubin fraction, the faeces containing no radioactivity and the urine only about 4% of that administered. The combined incorporation into liver, kidney and spleen was only 21%; the fate of the remaining radioactivity was not ascertained. The low incorporation into bile pigments of radioactivity from β-oxymesoferrihaem might have been due to low conversion into open-chain IXβ-tetrapyrrole or to inability of the rat to excrete the latter in the bile. Tritiated mesobilirubin was not available, but the excretion of tritiated mesobiliverdin-IXβ was studied since rats can rapidly convert biliverdin-IXα into bilirubin for excretion (Garay, Cantor, Argerich & Royer, 1964; Goldstein & Lester, 1964). In two experiments with mesobiliverdin-IXβ about 63% of the administered radioactivity was excreted in the bile in 24h. Excretion in the bile was rapid, 44% of the total radioactivity appearing within 2h of administration (Fig. 2). Although crystalline bilirubin was isolated in this experiment, its radioactivity was low and it was impossible to discover whether even small conversion of mesobiliverdin-IXβ into mesobilirubin had occurred.

Of radioactivity administered as α-oxymesoporphyrin, 63% was incorporated into bile and 75% of the total bile radioactivity appeared in the first 2h collection. The remainder of the radioactivity was excreted at a fairly steady rate during the subsequent 22h (Fig. 2). In the first and second 2h periods quantitative measurement showed bilirubin to be absent, and the green material isolated from the bile by the usual method for pigment isolation showed an absorption spectrum in chloroform ($\lambda_{max.}$ 405, 572 and 670nm) similar to that of the unchanged oxyporphyrin, some of which was therefore excreted unchanged. In a similar experiment with β-oxymesoporphyrin only 9% of the administered radioactivity entered the bile within 24h and this appeared mainly in the first 4h (Fig. 2).

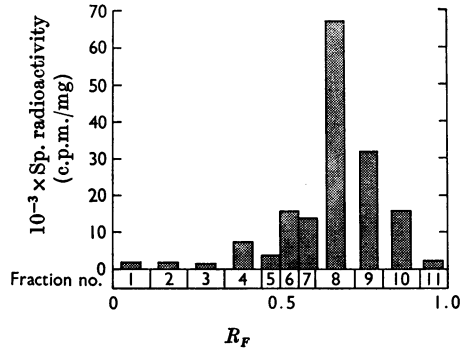


Fig. 1. Distribution of radioactivity into fractions from the t.l.c. of crystalline rubinoid pigments isolated from the bile of a rat to which tritiated α-oxymesoferrihaem was given intravenously. A sample (100μg) of the pigment in 0.1ml of chloroform was applied to Merck polyamide adsorbent and the chromatogram was developed in methanol–aq. 10% (w/v) ammonia–water (9:1:2, by vol.). Fractions numbered 6 and 7 (R_F 0.56) contained bilirubin; fraction 8 (R_F 0.67) contained mesobilirubin.

DISCUSSION

The present paper is concerned with the intermediates that might occur during catabolism of haem to bile pigments. The incorporation of radio-

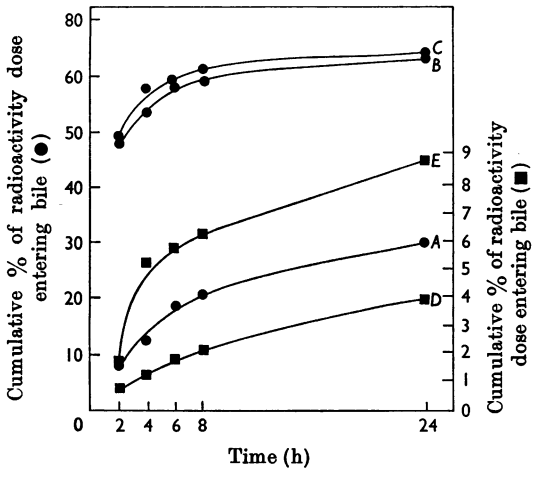


Fig. 2. Cumulative radioactivity excreted in the bile of rats during the first four 2h periods and the subsequent 16h after intravenous injection of tritiated α-oxymesoferrihaem (*A*), α-oxymesoporphyrin (*B*), mesobiliverdin-IXβ (*C*), β-oxymesoferrihaem (*D*) and β-oxymesoporphyrin (*E*). The left-hand ordinate refers to curves *A*, *B* and *C* (●); the right-hand ordinate refers to curves *D* and *E* (■). The quantities of tetrapyrroles used and their radioactivities are given in Table 1.

activity from injected α-oxymesoferrihaem into the bile of rats was high, with most present in the pigments. Fig. 1 shows the specific radioactivities of pigments extracted in this experiment and separated by t.l.c. As expected, the radioactivity was mainly in the mesobilirubin fraction of R_F 0.67. Small amounts of radioactivity were found in other regions of the chromatogram, probably owing to tailing.

The conversion of haem into an open-chain bile pigment in which two oxygen atoms replace the bridge carbon atoms of the haem is a process requiring at least 6 equivalents of oxygen. This is substantiated by Tenhunen *et al.* (1969), who showed that their enzyme preparation catalysed the production of bile pigments from haem with formation of carbon monoxide. The enzymic reaction utilized 12 equivalents of oxygen, of which 6 were directly concerned with porphyrin fission. A series of intermediates might therefore occur between haem and bile pigment, and oxyporphyrin derivatives might well represent a '2-equivalent' oxidation stage of catabolism.

Low excretion of radioactivity from β-oxymesoferrihaem and low incorporation into bile pigment was not due to the inability of the rat to excrete derived bile pigments because the corresponding verdin was rapidly excreted.

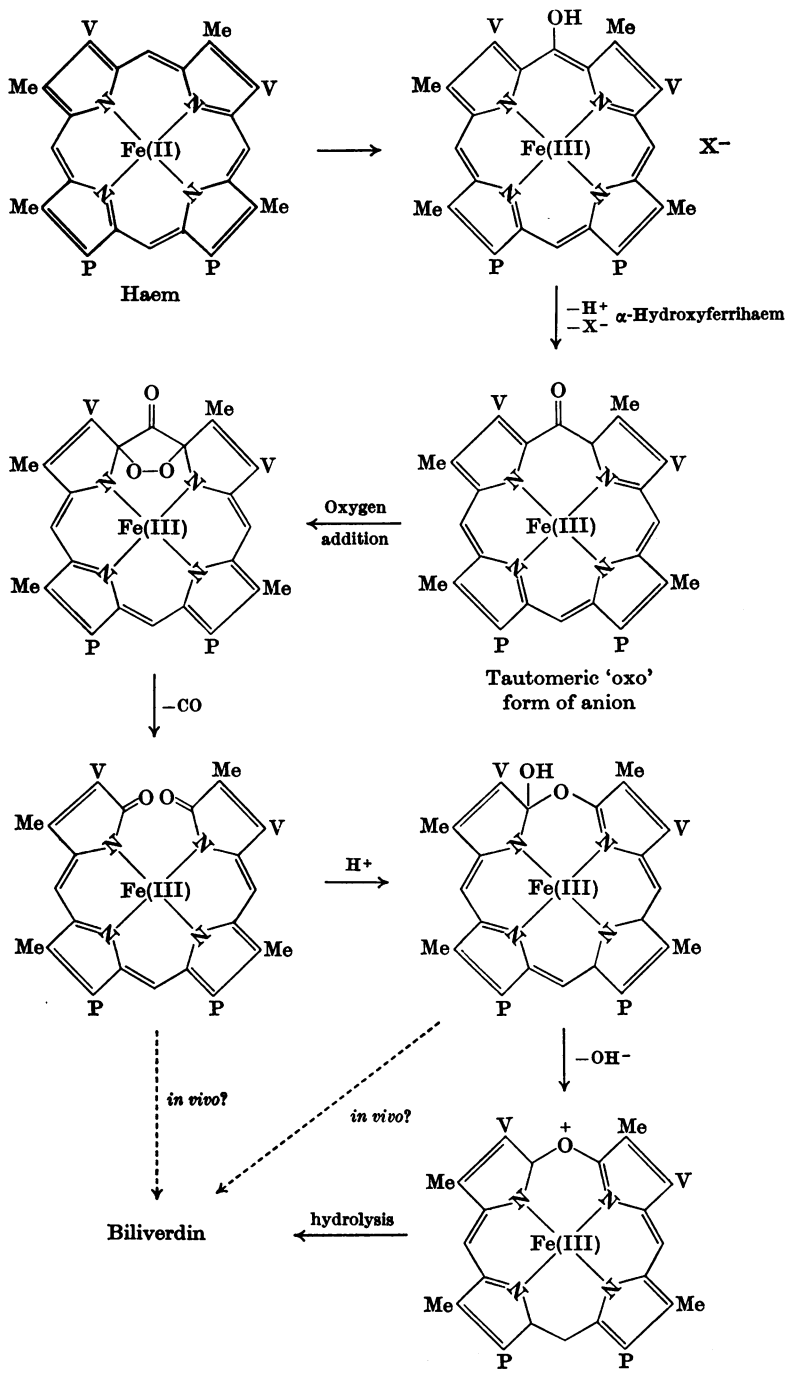
The failure to demonstrate conversion of α-oxymesoporphyrin or β-oxymesoporphyrin into bile pigment *in vivo* is consistent with the known stability of porphyrins compared with haems in oxidation experiments *in vitro* (Kench, 1954). Administration of α-oxymesoporphyrin was followed by the disappearance of bilirubin from the bile and the rapid appearance of radioactivity; bilirubin reappeared in the bile only when radioactivity had decreased.

The theoretical daily excretion of bilirubin in the rat calculated from a blood volume of 70ml/kg, an erythrocyte life-span of 70 days (Van Putten, 1958) and a contribution of 15% from non-erythropoietic sources, is 2700μg. Biliary excretion of α-oxymesoporphyrin, either intact or as a degradation product, calculated from excreted radioactivity was about 820μg. This excluded about 220μg of bilirubin from the bile in the first 2h. The 140μg of oxymesoporphyrin similarly calculated as present in the next 2h period equally inhibited bilirubin excretion. Only in the third 2h period, when bile radioactivity had fallen to a value corresponding to 138μg of oxyporphyrin, did bilirubin (160μg) reappear. The total bile bilirubin output of 1735μg occurring in the final 20h of the experiment was less than expected, and the discrepancy may be due to degradation by other routes or transfer through the gut wall of bilirubin excluded from the bile in the first 4h of the experiment. In spite of the approximations of such calculations the experiment showed that the administered α-oxymesoporphyrin excluded between one-quarter and one-half of a molecular proportion of bilirubin from hepatic excretion.

The high conversion of α-oxymesoferrihaem into bile pigments suggests that in the rat α-oxyprotoferrihaem may be the first intermediate between haem and bile pigment. This is in accord with current theories of haem breakdown and for the later stages we now put forward a modified version of the presently accepted scheme (Lemberg, 1956) involving direct addition of oxygen to the tautomeric oxo form of the oxyferrihaem anion (Scheme 1).

Both α-oxymesoferrihaem and β-oxymesoferrihaem in pyridine are oxidized by atmospheric oxygen to bile pigments (Jackson *et al.* 1968*b*), and therefore the labelled bile pigment obtained from the rat after injection of the α-oxy compound might possibly be a trivial artifact produced non-enzymically. The low conversion of the β-oxy compound renders this unlikely and indicates that the labelled mesobilirubin from α-oxymesoferrihaem is a product of metabolism.

Note added in proof. Since this paper was submitted R. Tenhunen, H. Marver, R. Schmid, W. Trager and D. Y. Cooper reported at the University of Aarhus Symposium of Bile Pigment Chemistry



Scheme 1. Hypothetical scheme for the conversion of haem into bile pigments showing the possible occurrence of α-monohydroxyhaem as a first member in a series of oxidized catabolic intermediates. Me = CH_3; V = $CH{=}CH_2$; P = $CH_2CH_2CO_2H$.

(August 1970) that bilirubin formed from haem in their liver microsome system in the presence of ^{18}O molecular oxygen incorporates 2 atoms of ^{18}O/molecule (mass spectrometric analysis). The bilirubin did not incorporate ^{18}O from $H_2{}^{18}O$. In the light of these results our scheme must be modified slightly as shown by the dotted pathways in Scheme 1. Verdoferrihaem is apparently not an intermediate in the process *in vivo* because otherwise the final bile pigment would contain only 1 atom of ^{18}O/molecule.

REFERENCES

Crook, P. J., Jackson, A. H. & Kenner, G. W. (1971). *Justus Liebigs Annln Chem.* (in the Press).

Garay, E. R., Cantor, D., Argerich, T. & Royer, H. (1964). *Medicina, B. Aires*, **24**, 22.

Goldstein, G. W. & Lester, R. (1964). *Proc. Soc. exp. Biol. Med.* **117**, 681.

Gray, C. H., Nicholson, D. C. & Nicolaus, R. (1958). *Nature, Lond.*, **181**, 183.

Jackson, A. H., Kenner, F. W., McGillivray, G. & Smith, K. M. (1968*a*). *J. chem. Soc.* C, p. 294.

Jackson, A. H., Kenner, G. W. & Smith, K. M. (1968*b*). *J. chem. Soc.* C, p. 302.

Kench, J. E. (1954). *Biochem. J.* **56**, 669.

Lemberg, R. (1956). *Rev. pure appl. Chem.* **6**, 1.

Lemberg, R. & Legge, J. W. (1949). *Haematin Compounds and Bile Pigments*, pp. 453–470. New York: Interscience Publishers Inc.

Malloy, H. T. & Evelyn, K. A. (1937). *J. biol. Chem.* **119**, 481.

Nakajima, H. (1963). *J. biol. Chem.* **238**, 3793.

Nakajima, H., Takemura, T., Nakajima, O. & Yamaoka, K. (1963). *J. biol. Chem.* **238**, 3784.

Ó Carra, P. & Colleran, E. (1969). *FEBS Lett.* **5**, 295.

Ostrow, J. D., Hammaker, L. & Schmid, R. (1961). *J. clin. Invest.* **40**, 1442.

Petryka, Z. & Watson, C. (1968). *J. Chromat.* **37**, 76.

Tenhunen, R. Marver, H. S. & Schmid, R. (1969). *J. biol. Chem.* **244**, 6388.

Van Putten, L. M. (1958). *Blood*, **13**, 789.