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Outstanding bile pigment problems

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Abstract

In this review of the chemistry, biochemistry and chemical pathology of the bile pigments I am well aware that I shall ask many more questions than I am able to answer. It seems appropriate that the subject should be considered under the five headings: -Chemistry; Formation from Haem Proteins; Transport in the Blood; Conjugation and Transport to the Bile; and Changes in the Gut. I shall conclude with a brief account of the early labelled bilirubin. Because investigation of the pathological significance of the chemical changes undergone in the body is possible only if the chemical structures of the bile pigments are accurately known, my department in London has been very much concerned during the last 15 years with the chemistry of the bile pigments. The work I shall describe has been carried out in collaboration with Dr. NICHOLSON, Dr. KULCZYCKA, Dr. COLE, Dr. PETRYKA and more recently by Mr. STOLL and Miss LEMMON.

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OUTSTANDING BILE PIGMENT PROBLEMS

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In this review of the chemistry, biochemistry and chemical pathology of the bile pigments I am well aware that I shall ask many more questions than I am able to answer. It seems appropriate that the subject should be considered under the five headings :-Chemistry ; Formation from Haem Proteins ; Transport in the Blood ; Conjugation and Transport to the Bile ; and Changes in the Gut. I shall conclude with a brief account of the early labelled bilirubin. Because investigation of the pathological significance of the chemical changes undergone in the body is possible only if the chemical structures of the bile pigments are accurately known, my department in London has been very much concerned during the last 15 years with the chemistry of the bile pigments. The work I shall describe has been carried out in collaboration with Dr. NICHOLSON, Dr. KULCZYCKA, Dr. COLE, Dr. PETRYKA and more recently by Mr. STOLL and Miss LEMMON.

Chemistry

As is well known, the bile pigments all possess a linear tetrapyrrolic structure with various numbers and arrangements of double bonds and various extents of the conjugation of those double bonds (Fig. 1). To this complexity there is added the complications caused by isomeric changes, such as by prototropy, whereby a bilirubin is readily converted to a biliverdin (1) (Fig. 2) and d-urobilin converted to a rhodin (2) (Fig. 3). We have been particularly interested in the structure of the urobilins in which only the two central pyrrole rings are linked by a double bond (Fig. 1) and our investigations have led to the isolation of two urobilins (3) additional to the i-urobilin, d-urobilin and stercobilin known to be present in faeces (Fig. 4). These new urobilins (Table 1) consist of an optically inactive form of d-urobilin and an optically active form of i-urobilin. Since then, WATSON (4) has described the isolation from faeces of a urobillin which resembled d-urobilin in its chemical reactions, crystalline form and melting point which was optically inactive, and a dl-stercobilin has been prepared by the catalytic hydrogenation of bilirubin (5). An optically inactive

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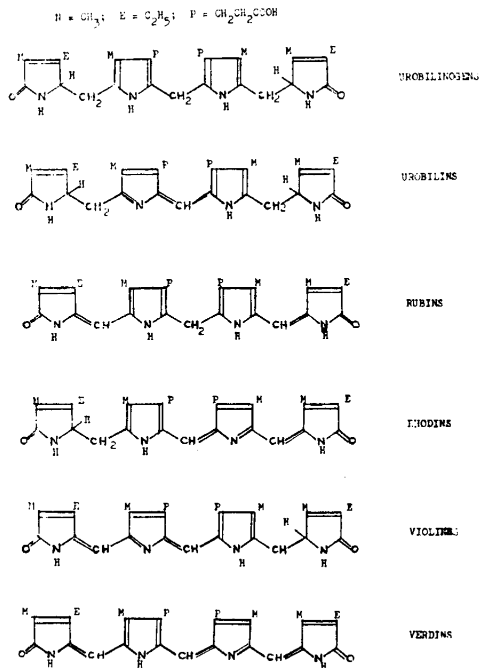
Fig. 1 Structures of the bile pigments



"PROTO" Pigments have vinyl groups instead of ethyl groups

Fig. 2 Possible mechanism of prototropy in bilirubin

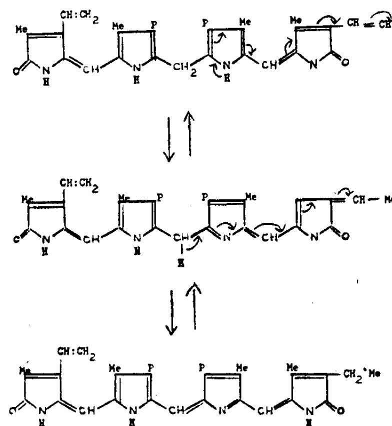


Fig. 3 Possible mechanism of prototropy in end ring of d-urobilin

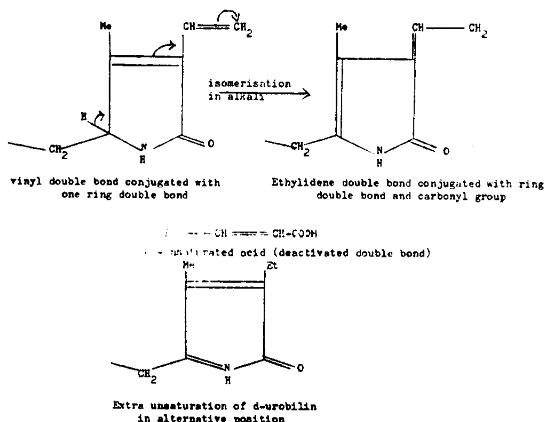


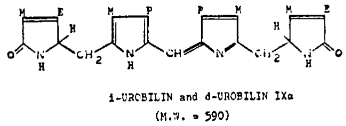
Table 1. Recently Isolated Urobilins

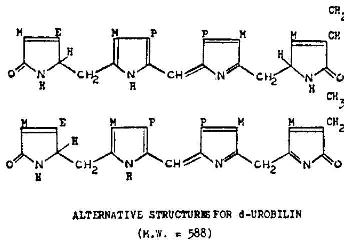
Pigment	Molecular Formula	Molecular Weight	Stability
Racemised d-Urobilin (3)	$C_{33}H_{40}N_4O_6$	588	Moderately stable
Racemised d-Urobilin (4) or d-Urobilin IXα	$C_{33}H_{40}$ or $_{42}N_4O_6$	588 or 590	Moderately stable
d-Urobilin IXα (3)	$C_{33}H_{42}N_4O_6$	590	Unstable
dl-Stercobilin (5) (catalytic hydrogenation of bilirubin)	$C_{33}H_{46}N_4O_6$	594	Stable
Tetrahydro-i-Urobilin (6) (synthetic)	$C_{33}H_{46}N_4O_6$	594	Stable

tetrahydro i-urobilin has been synthesised by PLEININGER and LERCH (6).

The cause of the high optical rotations of some urobilins is of particular interest because those of d-urobilin and stercobilin are among the highest known. We have studied the changes in optical rotation occurring on the formation of zinc complexes (7) (Fig. 5) and the resulting reduction in optical rotation leads us to believe that the optical rotation is due to the helical shape of the molecule which was made more planar by formation of the zinc complex. The behaviour of stercobilin on complex formation, however, was anomolous (Fig. 6) in showing a series of changes in optical rotation which until recently have defied explanation. However, a closer examination of the changes in the optical rotatory dispersion spectrum on zinc complex formation has revealed that this behaviour can be explained entirely by the changes during complex formation in the shape of the curve relating optical activity to wavelength (Fig. 7). The optical rotatory dis-

Fig. 4 Currently accepted structures for the "CLASSICAL" urobilins





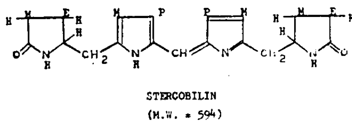


Fig. 6 Change of optical activity of stercobilin hydrochloride in chloroform on addition of zine acetate

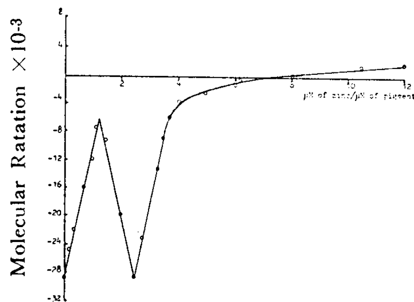


Fig. 5 Change of optical activity of d-urobilin hydrochloride in chloroform on addition of zinc acetate

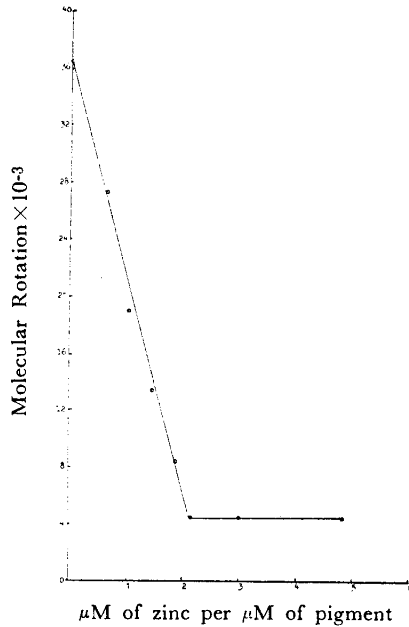
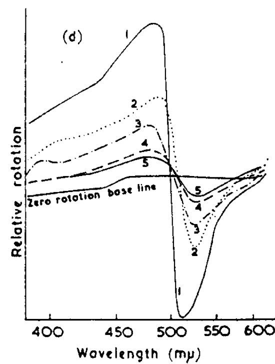


Fig. 7 Optical rotatory dispersion by chloroform solution of stercobilin hydrochloride in the presence of increasing amounts of zine acetate (1) without zinc acetate (2) with 1.2 mols. of zinc acetate (3) with 2.1 mols. of zinc acetate (4) with 7.9 mols. of zinc acetate (5) with 13.1 mols. of zinc acetate.

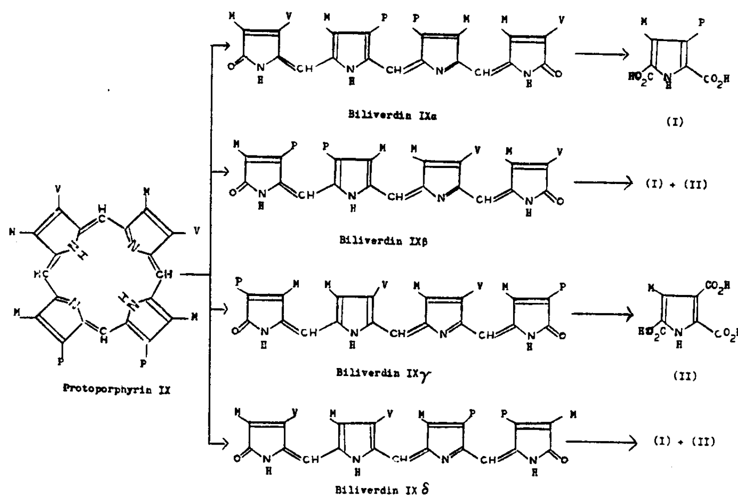


persion curve of stercobilin when 1.2 moles of zinc acetate are added crosses that with 2.1 moles of zinc acetate added at a wavelength of about 550 mμ. At 589 mμ (the mean wavelength of the sodium lines) the optical rotation falls with the addition of about 1 atom of zinc. The rotation then returns to approximately its original value on the addition of a second atom of zinc and then progressively falls on the addition of further amounts of zinc.

We had at one time hoped that mass spectrometry of the bile pigments would throw light on their structure and indeed in an earlier paper (8) we believed that we had demonstrated that stercobilin was a double molecule, which would have explained many earlier observations. However, it is now known that stercobilin is split into two components under the conditions of temperature and pressure in the mass spectrometer, and that our earlier interpretation of this change was erroneous.

We were interested in the problem of isomer types of bile pigments (Fig. 8) and NICHOLSON, NICOLAUS and I showed that all naturally occurring bile pigments were predominately IXα in type, i. e. they were formed from protoporphyrin by rupture at the α-methene bridge (9). The identification of isomer type depends on the specific mixture of monopyrrolic acids formed on oxidation with permanganate. However, Dr. PETRYKA (10, 11), first in my department and later confirming his work in Minneapolis, has shown that early-labelled stercobilin may contain a small amount

Fig. 8 Isomeric verdins theoretically formed by fission of protoporphyrin IX, and their oxidation products the pyrrolic acids (I) and (II). $M=CH_3$; $V=CH:CH_2$; $P=CH_2 \cdot CH_2 \cdot CO_2H$

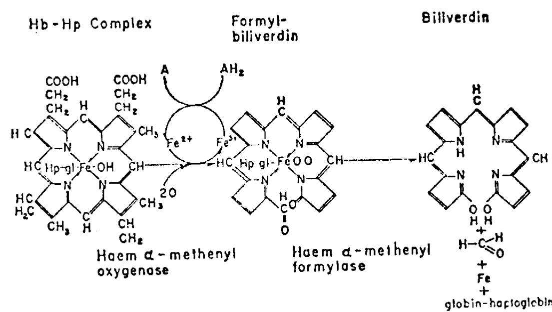


of an isomer other than the IXα form and this has recently been supported by the work of Miss Lemmon in my laboratory. She has developed a more sensitive gas chromatographic method for the detection of the pyrrolic acids formed on oxidation. It seems likely that the weakness of the NICOLAUS (12) method of investigations is the very low yield of pyrrolic acids formed by oxidation and there is, therefore, a probability that small amounts of isomers, other than the IXα type, would not be detected, in that insufficient pyrrolic acids would be formed for identification. There is urgent need for a more thorough re-investigation of this method.

Formation of Bile Pigments from Haem Proteins

I pass now to consider the formation of bile pigment from haemoglobin and other haem proteins. It would be presumptuous of me to describe in detail the major advances made in this country concerning the action of haem α-methene oxygenase in forming formylbiliverdin (13, 14) (Fig. 9). It

Fig. 9 Possible mechanism of haemoglobin degradation via haemoglobin-haptoglobin complex.



was the profound importance of this work that inspired me to arrange for Dr. Osamu NAKAJIMA to visit my department to establish the IXα nature of the product of this enzymic pathway. Although MURPHY, O'HEOCHA and O'CARRA (15) in Ireland and more recently LEVIN (16) in the United States have found difficulty in preparing an active enzyme, Dr. NAKAJIMA has repeated his work and we have succeeded in showing that the bile pigment formed is mainly, if not exclusively, IXα in type (17). It is our belief that these workers have been unable to prepare an active liver extract and Professor O'HEOCHA is sending one of his colleagues to our department to prepare such an extract under Dr. NAKAJIMA's guidance. Dr. NAKAJIMA himself has also become interested in elucidating the true substrate of the oxygenase and he is at present examining the substrate activity of the sub-units of haemoglobin and of methaemalbumin. It will

also be of importance to examine the activity in this respect of haem proteins such as cytochrome P_{450} which has received so much attention recently. Of particular interest is that the highest oxygenase activity is present in the kidney; Dr. NAKAJIMA has found that haemolysis induced by phenylhydrazine increases the haem α-methene oxygenase activity in the liver by three times thus raising it to the same level as that observed in the kidney. We are hoping in the near future to examine especially the high activity in the kidney in respect of the cytochrome P_{450}. It seems very likely that this may be an important source of bile pigment formation (18).

Transport to the Liver, Conjugation and Transport to the Bile

Passing to the problems of transport, conjugation and excretion, I do not intend to consider these in detail, although there are a number of fascinating problems, especially in relation to the disaggregation of the bilirubin-protein complex in passing from the blood plasma to the liver cell, the transport of the bile pigment to the site of conjugation and the transfer of the conjugated material to the bile. Much work has been done in this country and by Dr. BILLING (19) and by Professor SCHMID (20), both of whom have recently visited Japan and have told you of their important work in these fields. There are, however, a number of outstanding problems. What is the abnormality in the various forms of non-haemolytic retention jaundice, not all of which seem to be due to an enzyme deficiency? Furthermore, there is need for further investigation into the behaviour of the biliverdin reductase, which is clearly of importance in reduction of biliverdin to bilirubin, the form in which the bile pigments are transferred from the reticulo endothelial system to the liver.

Dr. Roger WILLIAMS and Dr. Richard THOMPSON of my medical school are investigating some of these aspects. They have been particularly interested in examining the mode of action of phenobarbital on the serum bilirubin. The first observation in this field was that of CATZ and YAFFE (21) who showed an increased liver glucuronyl transferase in mice treated with phenobarbital. YAFFE and, independently, CRIGLER and GOLD (22) then observed a reduction in the serum bilirubin concentration on giving phenobarbital to children with the Crigler-Najjar syndrome, while GARTNER (23) could find no change in the serum bilirubin of Gunn rats treated for two months with the drug. SCHMID (18) investigated the effect on the incorporation of labelled glycine into the biliary bilirubin in untreated rats and rats treated with phenobarbital. There was increased synthesis of the drug metabolising enzymes cytochrome b_5 and P_{450}. He observed a considerable increase in the early labelled fraction. Recently, Dr. WILLIAMS

and Dr. THOMPSON (24) have studied the effect of phenobarbital on the serum bilirubin of 4 patients with biliary cirrhosis of long standing. In these, chronic cholestasis was prominent. In all, phenobarbital treatment resulted in a fall in the plasma bilirubin. In these patients the plasma bilirubin had been relatively constant at a raised level for nearly a year. It is difficult to understand the significance of these contradictory findings and although one is tempted to speculate on the relationship between these effects of phenobarbital on plasma bilirubin on induction of mitochondrial cytochromes and in inducing acute attacks in patients with acute porphyria, there is urgent need for much more work in this field.

Changes in the Gut

The bile pigments are excreted in the bile into the duodenum in conjugated form and the changes occurring during its passage down the gut include hydrolysis, hydrogenation, dehydrogenation and degradation of tetrapyrrolic structure (25). Only some of the resulting substances are absorbed from the gut. Because the chemical, physical and biological properties of free and conjugated bilirubin are very different, the fate of bile pigments in the gut and other changes they undergo must depend upon the extent of hydrolysis of conjugated bilirubin and upon the site of that hydrolysis. The ease with which bilirubin glucuronide is hydrolysed and the known presence within the gut of bacterial and intestinal glucuronidases suggests that the free pigments might be liberated from glucuronides in the intestinal tract. It is not yet known whether the conjugates are hydrolysed before or after reduction, although a quantitative conversion of bilirubin glucuronide to urobilinogen but not of bilirubin is brought about *in vitro* by normal faecal flora in broth cultures (26). A little bilirubin normally remains unreduced in the intestine and is excreted in the faeces but it is not known if this is conjugated or not. Since, however, urobilin and urobilinogens are the main faecal tetrapyrrolic pigments excreted, considerable quantities of bilirubin glucuronide probably normally reach the colon.

The conversion of bilirubin in the gut to the final products, the urobilinogens, is generally accepted as a stepwise reduction effected by dehydrogenases of the intestinal bacteria (27), but surprisingly little is known of the detailed course of the reaction. Outstanding problems concern the role of the glucuronide radical, the role of co-factors in the bile, the order with which and the sites at which the various pigments arise and whether reduction alone explains their formation. The proportions of i-urobilin and d-urobilin and stercobilin which can be obtained from the

faeces are quite unpredictable and are independent of disease (28). The absorption of urobilinogen in the colon, its normal return to the liver with re-excretion in the bile constitutes the enterohepatic circulation first postulated by MÜLLER (29) and repeatedly reviewed since.

Urobilinogen absorbed from the intestine is excreted in the urine in large amounts in the early and in the recovery stages of hepatogenous jaundice. Early demonstrations of the enterohepatic circulation believed to be responsible for this depended on such experiments as the appearance of urobilinoid substances in the bile after enemata, containing mesobilirubinogen, had been administered to patients with biliary fistulae. Recent methods by SCHMID and his colleagues for the production of isotopically labelled bilirubin (30) and of mesobilirubinogen (31) have permitted unequivocal demonstration of the intestinal absorption of mesobilirubinogen but not of urobilin. There is also absorption of bilirubin but not of conjugated bilirubin (32, 33). Absorption and enterohepatic circulation of [^{14}C] mesobilirubinogen have been demonstrated after introduction of the labelled pigment into either the terminal ileum or the duodenum of rats and in man (31, 34). There is slower absorption from the terminal ileum than from the jejeunum or duodenum and this may be the result of the reduced surface available for absorption as well as of adsorption of the urobilinogen on the faecal solids. Urobilinogenuria has long been regarded as resulting from impaired re-excretion into the bile due to liver disease, to biliary trauma and infection. It is possible that the presence of liver disease may permit invasion of the ileum by micro-organisms and of urobilinogen formation which may occur higher in the intestine than normal. Absorption is more rapid and complete in this part of the gut so that urobilinogenuria may reflect enhanced intestinal chromogen absorption as well as impaired hepatic excretion (31). Infection of the biliary tract by certain micro-organisms capable of forming d-urobilin from bilirubin glucuronide is associated with d-urobilinogenuria and this may be due to the spread of these organisms up the gut. d-urobilinogen may thus be due to absorption of this chromogen from the gall bladder itself or from the upper part of the small intestine. LESTER and SCHMID have successfully shown that in both rats and in man absorption of bilirubin can occur throughout the entire length of the small intestine and of the colon (32, 33). In the human there seems to be less intestinal hydrolysis of conjugated bilirubin than in rats so that in normal man little enterohepatic circulation of bilirubin occurs. In human subjects with the congenital defect of hepatic bilirubin conjugation, crystalline labelled bilirubin was isolated in considerable quantity from the stools after injection of [^{14}C] bilirubin (35). It

must, therefore, be concluded that bilirubin can pass also from blood through intestinal mucosa of the lumen. This is an excretory route which possibly occurs normally to a minor extent but is of especial value when hepatic excretion is limited. An attempt has been made unsuccessfully to increase excretion in subjects with defective conjugation of bilirubin by this route, by the oral administration of cholestyramine (36). This is a resin which can absorb bilirubin.

For many years my department has been concerned with the precise chemical structure and estimation of the faecal bile pigments and we are now in complete agreement with C. J. WATSON and his group that the proportions of the three urobilins and of their colourless precursors are entirely fortuitous and dependent on diet and intestinal flora (28). Nevertheless, there remains the problem of the colour of normal faeces; this has recently been raised by WITH (37) at a conference in London.

Closely related to this problem is the pathway of elimination of bile pigments in the Gunn rat and in the Crigler-Najjar syndrome. Both SCHMID (35) and BILLING and I (38) have found that labelled bilirubin is in part degraded to substances other than the known bile pigments. In this connection, a study of the yellow pigment found in the bile of Gunn rats and in the bile of these subjects would be worthy of investigation.

The Early Labelled Bilirubin

The problem of the early-labelled bilirubin has become extremely complex since London and his colleagues in New York (39) and my own group in London (40) showed that a proportion of the bile pigment was formed from a source or sources other than the haemoglobin of erythrocytes at the end of their life span. This early-labelled bile pigment is produced in excess in the following pathological conditions: pernicious anaemia, sickle cell anaemia, thallassaemia, aplastic anaemia, congenital porphyria, shunt hyperbilirubinaemia, coproprotoporphyria. Subsequent work by SCOTT and me (41) and by others showed that this bile pigment fraction labelled during the first 10 days after administration of isotopically labelled glycine was increased by increased erythropoiesis (42).

Professor YAMAMOTO with ISRAELS and their colleagues showed that labelled bilirubin appeared within about 1 hr. and persisted for 4—6 days in the bile of dogs with biliary fistula (43). They were therefore the first to shown how rapidly this fraction was synthesised. Later they showed that this early-labelled fraction must consist of at least 2 components (44, 45). The first of these components appeared in the plasma within 90 minutes and lasted 24 hr.: the other did not reach a maximum until 3—5

days after the glycine. This second component, the so-called erythropoietic component, was affected by changes in the rate of erythropoiesis and its time of appearance approximated to that expected for the incorporation of [^{14}C]-glycine into the maturing red cell. The first component was not affected by erythropoiesis and was thought to derive from a source other than the bone marrow. Such a double or multiple origin of the early-labelled pigment is better shown by changes in isotope incorporation into the bile pigment of the plasma or of the bile rather than into that of the faecal stercobilin, because the faecal bile pigment can often be isolated only from pooled collections of faeces. Nevertheless, we ourselves have noticed during isotope experiments that double peaks sometimes occur during the first few days in the labelling of faecal stercobilin but have hesitated to attribute significance to such double peaks because of the possibility of experimental error. Such a double peak was found with a child with congenital hyperbilirubinaemia, who we described in a paper published with Dr. Billing (38). We have, however, unequivocally confirmed the existence of the double peak in the labelling of faecal stercobilin in an adult patient with congenital hyperbilirubinaemia.

As shown by Professor Yamamoto, Israels and their colleagues the erythropoietic and non-erythropoietic components can be distinguished by the use of isotopically labelled δ-aminolaevulic acid (ALA) since this precursor is poorly incorporated into haem and therefore poorly incorporated into the erythropoietic component of bile pigment but rapidly incorporated into the non-erythropoietic fraction (46). My own group with that of Rimington has shown that in erythropoietic protoporphyria a substantial proportion of the excreted protoporphyrin became labelled at the same time as the early-labelled fraction of bile pigment and that there was a strong possibility that the non-erythropoietic component was derived from hepatic haems (47). As stated in our publication, the amounts of stercobilin, coproporphyrin and protoporphyrin isolated from the faeces each day were sufficient to demonstrate a considerable excess of early labelled bile pigment. The maximum labelling of these three compounds occurred at the same time so that this bile pigment fraction must have been formed with the faecal coproporphyrin and protoporphyrin from a common precursor. Unfortunately only small amounts of protoporphyrin could be isolated from the red cells necessitating considerable dilution of the isolated material to permit measurement of the ^{15}N content. Only 3 measurements were significantly different from the zero excess of ^{15}N. We have recently repeated this experiment with a second subject and using a more sensitive mass spectrometer, and have now convinced ourselves that the isotope content

of the free protoporphyrin in the red cells is at the most one-twelfth that of the faecal protoporphyrin. We feel therefore that in this condition a substantial proportion of the faecal porphyrin and of the early-labelled bile pigment must be derived from a source other than the maturing red cells and that this source is very probably the liver (48).

The Minneapolis group led by SCHWARZ have shown that in biliary fistula dogs, much of the early-labelled bilirubin could be accounted for by the turnover of label in the easily-split haem of liver and to a lesser extent of the kidney (49). Moreover, SCHMID and his group have confirmed a hepatic origin of part at least of the early-labelled bile pigment and have shown that the perfused isolated liver can synthesise bilirubin from ALA (50).

Recently ISRAELS and his colleagues have studied the incorporation of [^{14}C]-ALA into the bilirubin of the bile and into the hepatic and kidney haems in biliary fistula rats (46). They obtained convincing evidence that a fraction of the labelled bilirubin was synthesised within a few minutes of the injection of the ALA and that this very early-labelled fraction was unlikely to be related to the turnover of those haem fractions of the liver and kidney which had hitherto been investigated. On the other hand, a substantial proportion of the labelled bilirubin excreted between 2 and 24 hr. after injection was probably derived from the turnover of the major haem proteins of the liver and kidney.

These findings strongly suggested that the early-labelled bile pigment consists of three fractions. The early-labelled pigment, in addition to the erythropoietic fraction, consists of 2 other components, one certainly derived from the turnover of the major haem proteins of the liver and kidney and a second component formed too rapidly to be accounted for by catabolism of the major haem proteins. However, even in liver homogenates incubated with [^{14}C]-ALA, newly synthesized haem and bilirubin appeared within 5 minutes and this may be accepted as evidence that the very early bilirubin is derived from a haem protein with an unusually rapid turnover.

The late non-erythropoietic fraction may well be derived from liver catalase or cytochrome, the half-lives of which are about 24 hr. but the rapidly-turning over haem protein responsible for the very early non-erythropoietic fraction is less easily identified. This very early bilirubin which is most readily detected in the plasma might well be derived from some other organ than the liver for it is difficult to accept that it should be secreted into the blood stream by the liver only to be re-excreted by that organ. In this connection, one wonders if the kidney were the organ

concerned, especially since the highest activity of the enzyme haem α-methene oxygenase is found in the kidney at any rate of the guinea-pig. However, there may be species differences in the proportions of the three fractions of the early-labelled bile pigment. Indeed there is some evidence that the non-erythropoietic components may be less significant in normal man than in some experimental animals such as the rat and the dog. Nevertheless, the earlier observations have been extended to human subects (45) in whom, after [^{14}C]-ALA, the specific activity of plasma bilirubin showed a single peak at 1—2 hr. while bilirubin isolated from bile collected with a T-tube in the common bile duct exhibited its peak at 6—12 hr. but maintained a shoulder at 12—24 hr.

There then seems little doubt that the early labelled bile pigment is obtained from at least 3 sources:

(i) in association with erythropoiesis
(ii) from rapidly turning-over haem such as liver cytochromes or catalase
(iii) from very rapidly turning-over haem perhaps in extrahepatic tissues as well as in the liver.

The source of this last fraction is of particular interest. Schmid has produced some evidence that it might be derived from the mitochondrial cytochrome P_{450}. This is associated with the smooth part of the endoplasmic reticulum and the levels and turnover rate are more than sufficient to account for this fraction of the early-labelled pigment. Schmid has found when rats are treated with phenobarbital to induce increased amounts of this enzyme, the very early labelled peak is greatly increased. This provided strong evidence that the very early labelled fraction might be derived from this cytochrome, but other effects of barbiturates, such as in biliary cirrhosis, are more difficult to explain.

There is urgent need to investigate bilirubin metabolism before and during phenobarbital therapy using labelled bilirubin. There is also need to investigate the contributions made by the three early-labelled bile pigment fractions to the total bile pigment production. In this connection, similar investigation could be well worth while in all the conditions in which the early-labelled bile pigment is increased. It has alway been assumed that the early-labelled pigment in erythropoietic or congenital porphyria is erythropoietic in origin. Is this so? In erythropoietic protoporphyria the peak of labelling of faecal stercobilin is very sharp at 4 days. May this be the second non-erythropoietic fraction? It may well be. All the other conditions require similar investigation using [^{14}C]-ALA, but will not be really practical until more sensitive methods are divised for the

measurement of the specific activity of plasma bilirubin in the absence of hyperbilirubinaemia.

There are many other problems in need of answers. What is the cause of the increased proportion of early-labelled bile pigment in extra hepatic biliary obstruction? Why have double peaks been observed in the labelling of faecal stercobilin in congenital hyperbilirubinaemia and only seldom in other human subjects? Might this condition be due to passage to the liver of increased amounts of bile pigments produced in other organs such as the kidney? These are matters my department is at present investigating.

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