Defects in bile pigment metabolism causing jaundice

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Abstract

The excretion of bilirubin and therefore the relief of jaundice is dependent upon at least three factors. First, the bilirubin must be conjugated and thus converted into a water soluble compound: this means its conversion to an ester glucuronide although other conjugates may also be formed. Secondly, there is the problem of the transport of bilirubin through the hepatic cell. A defect in either the up-take of bilirubin or the secretion of conjugated bilirubin may result in jaundice such as is seen in the various types of familial hyperbilirubinemia. Thirdly, there is the possibility of alternative catabolic pathways for bilirubin: this approach to the problem has, however, not yet received the attention of investigators.

DEFECTS IN BILE PIGMENT METABOLISM
CAUSING JAUNDICE*

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It gives me very great pleasure to be here in Okayama. I have long known of the very excellent work that has been done in this University in connection with bile pigments so that it has been a wonderful experience for me to have the opportunity of talking with Professor Kosaka, Dr. Sakamoto and others of this department.

Today I am going to talk about some of the defects in bilirubin metabolism which may cause jaundice. It has been known for over forty years that for bilirubin to be excreted in the bile and urine, it must first be converted from a lipid-soluble pigment into one which is water-soluble. This raises the question as to what is the nature of this water-soluble pigment. It is due to Professor Kosaka and Professor Yamaoka[1] that the first real clue to this problem was obtained. They were able to show that the excretion of bilirubin involves its conversion into an ester. It was at this stage that Dr. Lathe, Mrs. Cole and myself at Queen Charlotte's Maternity Hospital in England started to investigate the problem.

We have employed the technique of reverse phase chromatography using silicone treated kieselgur and with a chloroform, carbon tetrachloride, methanol solvent system at pH 6.0 were able to separate the bile pigments into two components, namely bilirubin and a second component which gives the direct van den Bergh reaction, in the absence of protein and bile acids[2]. If the solvent system is changed to butanol and water the direct reacting pigment can be resolved into two components namely, pigment I and pigment II (Fig. 1)[3]. Pigment II is the main pigment in bile, while both pigments I and II can be found in serum and in urine. We then tried to isolate and identify these pigments but they are extremely unstable, and so we had to tackle the problem in a different way. Kawai[4] in this hospital and myself had independently observed that if you examine the products of the van den Bergh reaction on icteric serum that bilirubin gives you one azo pigment (azo pigment A) whereas pigment II gives you a different one (azo pigment B). Using reverse phase chromatography,

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* The paper read at Okayama University Medical School, Okayama, Japan, Sept. 14, 1960.
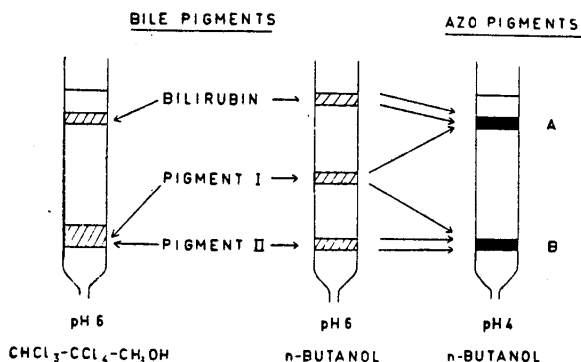
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Fig. 1. Behaviour of bile pigments and their diazo products on reverse phase partition chromatograms (Nosslin, B.: Scand. J. Clin. Lab. Invest. 12, Suppl. 49, 1960).

paper chromatography, or counter-current distribution techniques it is possible to separate azo pigments A and B, both of which are relatively stable. We were able to isolate azo pigment B[5], as did Dr. Schmid[6] in the United States, and found that azo pigment B is the ester glucuronide of azo pigment A (Table 1).

Table 1. Identification of Pigment II.

IDENTIFICATION OF PIGMNT II

1. Pigment B $\xrightarrow[\beta\text{-glucuronidase}]{\text{alkali}}$ Pigment A + glucuronic acid

2. I mol Bilirubin $\longrightarrow$ 2 mols Pigment A

THEREFORE

I mol Pigment II $\longrightarrow$ 2 mols Pigment B

Conclusion : Pigment II = ester diglucuronide of bilirubin

Evidence for this statement is based on the observation that when azo pigment B is treated with mild alkali or with the enzyme β-glucuronidase, pigment A and glucuronic acid are formed. Now, Overbeek and his collaborators[7] had shown that one molecule of bilirubin will give two molecules of azo pigment A. We, therefore, presumed that one molecule of pigment II would give two molecules of azo pigment B and concluded that pigment II is the ester diglucuronide of bilirubin (Fig. 2), the glucuronyl radicals being attached through the proprionic groupings of the bilirubin molecule. Pigment I forms both azo pigment A and
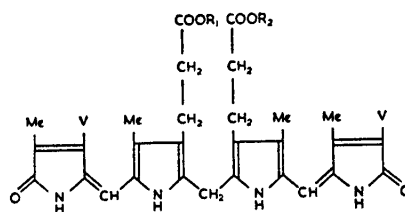
Structure of bile pigments (Bilirubin, R_1 and R_2 = H; pigment 1, R_1 = glucuronyl and R_2 = H, pigment II, R_1 and R_2 = glucuronyl; in all, Me = methyl and V = vinyl)

Fig. 2.   Structure of bile pigments (Billing, B. H.: Advances in Clinical Chem. 2, 267, 1960).

azo pigment B in the van den Bergh reaction and appears to be bilirubin mono-glucuronide.

Although the main means of excreting bilirubin involves conjugation as the glucuronide, other detoxifying mechanisms may be employed and must be considered. Mild alkali treatment of icteric serum (or bile) will convert most, but not all, of the conjugated ("direct") bilirubin into bilirubin; the remaining conjugated bilirubin has been shown to have a non-glucuronide structure. The possibility that this might be bilirubin sulphate was examined by Isselbacher and McCarthy[8] in America (Fig. 3). They injected radioactive S^{35} into a rat and
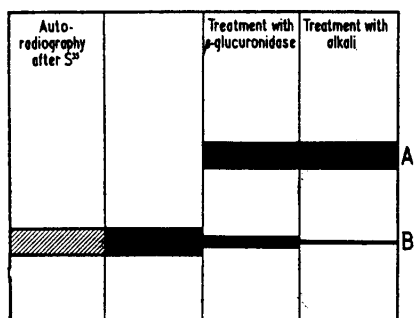

Fig. 3. The chromatographic behaviour of purified azo pigments obtained from rat bile after the injection of radioactive sulphate, and after treatment with β-glucuronidase and alkali (O. IN NaOH). The corresponding autoradiograph shown on the left indicates that the radioactivity directly coincides with the azo-pigment B (drawn from data given by Issselbacher and McCarthy, 1959). (J. Clin. Invest. 38, 645, 1959)

collected the bile. After diazotisation the resulting azo pigment B was isolated by chromatography and autoradiograms showed that it contained S^{35}. In addition, Dr. Kondo in this department has demonstrated that some of the bile pigments may be excreted as ester phosphates and there is some evidence that glycine conjugation may also occur.

An examination of the distribution of the 3 bile pigments in different types of jaundice shows that bilirubin is the main pigment in hemolytic disease, both in the adult and in the new born, and also in the premature infant (Fig. 4).
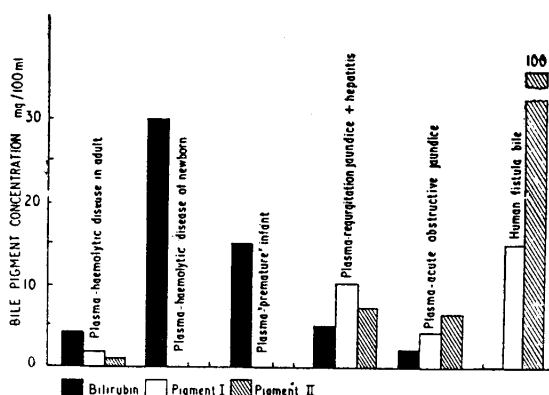
Fig. 4.   Examples of the relative amounts of bilirubin pigment I and pigment II found in plasma in various conditions and in fistula bile. (Billing, B. H. + Lathe, G. H. Amer. J. med: 24, 111, 1958).

In bile, as previously mentioned, there are two pigments, pigment II and some pigment I, so that if there is an obstruction to the flow of bile, one would expect that these two pigments would accumulate in the blood. Thus in obstructive jaundice, the pigment that accumulates most is pigment II, and there is also some pigment I and bilirubin. On the other hand, in hepatitis, or hepato-cellular disease there is more pigment I than pigment II. In other words, I think, there is probably a defect in the conversion of the monoglucuronide to the diglucuronide. The different pictures seen in these two types of jaundice suggested that a comparison of the relative amounts of pigment I and pigment II might prove to be a useful diagnostic test for the differentiation of obstructive jaundice and hepatocellular disease.

Dr. Bollman and his colleagues at the Mayo Clinic[9] examined over 100 sera and found that in hepato-cellular disease less than 50 per cent of the conjugated pigment is pigment II i. e. pigment I is the dominant pigment (Fig. 5). In biliary obstruction, however, there is more than 50 per cent of the conjugated pigment as pigment II. Unfortunately there is some overlap in the results and the chromatographic technique is not sufficiently accurate to make this a good test.

Bilirubin glucuronide is formed by the action of a specific enzyme, a glucuronyl transferase with uridine diphosphate glucuronic acid as the glucuronyl donor (Fig. 6)[10,11] The glucuronyl transferase is found mainly in the liver microsomes and to a small extent in the kidney and intestinal tissue. At present, however, there is no evidence that tissues other than the liver are taking part in the synthesis of bilirubin glucuronide *in vivo*.
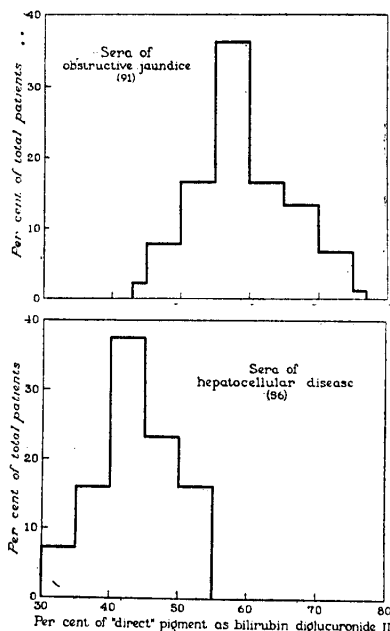
Fig. 5. Distribution of direct-reacting pigment II in the serum of patients suffering from liver disease. (Billing, B. H.: Advances in Clin. Chem. 2, 267, 1960)
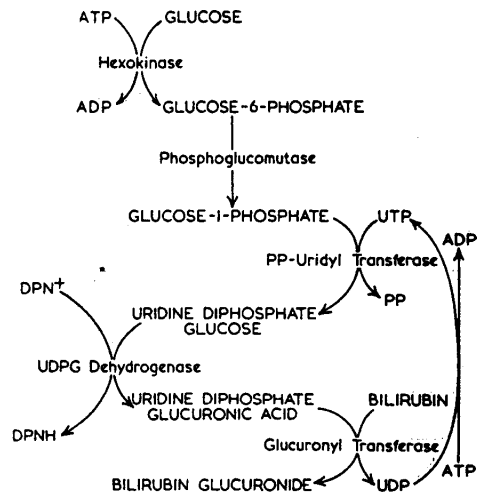

Fig. 6. A possible mechanism for the conjugation of bilirubin with glucuronic acid. (Billing, B. H and Lathe, G. H. Amer. J. med, 24, 111. 1958)

A deficiency of glucuronyl transferase would be expected to cause an accumulation of bilirubin in the body and such is the position in the new born baby. It can be shown in animal experiments and also from the analysis of liver tissue obtained from new born babies that there is a relative or absolute deficiency of glucuronyl transferase at birth.

Examination of the changes in plasma bile pigment concentration during the neonatal period shows that the highest levels of bile pigment concentration are attained in the smallest babies (Fig. 7). Also, the smaller the baby the longer it takes to reach the maximum level. Thus, with a six pound baby the highest bile pigment concentration may be seen in four days, whereas with a three pound baby it may take seven days. This is due to the fact that the very small baby has a less mature liver and therefore there is less glucuronyl transferase. As soon as sufficient glucuronyl transferase has developed then the baby will recover, since the bilirubin can then be excreted as bilirubin glucuronide and removed in the bile in the normal way.

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In hemolytic disease in the new born, the trouble is two fold. First of all, the infant is receiving an increased load of bilirubin due to the hemolytic process and then secondly, there is this problem of hepatic immaturity. The concentration of bilirubin in the plasma will therefore rise very quickly and attain a very much higher level. Unless exchange transfusion therapy is instituted quickly the plasma bilirubin may reach dangerous levels so that the bilirubin may go into the brain causing kernicterus.
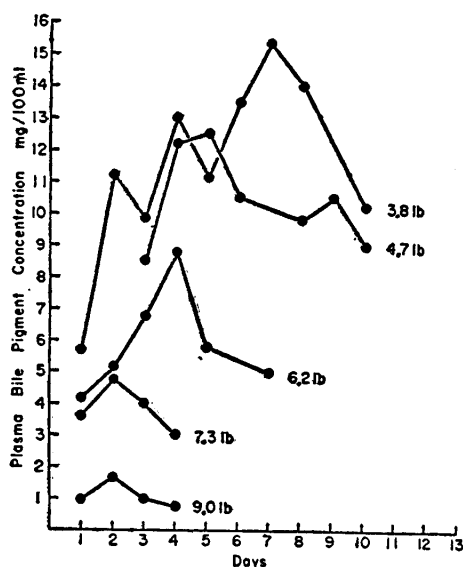

Fig. 7. The course of plasma bile pigment concentration during the neonatal period of five typical babies of varying birth weights (Billing et al. Brit. Med. J. ii, 1263—1265, 1954).

The toxicity of bilirubin has been shown by *in vitro* experiments, in that it will uncouple oxidative phosphorylation. It will also decrease the oxygen consumption of brain brei, a process which can be reversed by adding cytochrome C, methylene-blue or DPN to the mixture. Exactly how the bilirubin acts, *in vivo* is uncertain but it is obvious that it will upset the metabolism of the brain tissue. Very high doses of bilirubin given to animals will also cause neurological damage.

Conjugated bilirubin on the other hand does not appear to be toxic so that in adults brain damage is not associated with severe hyperbilirubinemia since the high levels of pigment are due mainly to conjugated bilirubin and not indirect bilirubin. It is very rare that brain damage results in infants unless the bilirubin concentration in the serum goes above 20 mg%. But there are occasions when it has been reported that brain jaundice results from very much lower levels. Several years ago, in the United States in a premature nursery[13], the babies were given two different types of antibiotic treatment, one group received oxytetracycline and the other group received gantrisin (a sulfa drug). 44 of the 95 babies who received the gantrisin treatment died. In the other group, of similar size, receiving oxytetracycline therapy only 22 died. The difference in the number of deaths was due to a very high incidence of kernicterus. The increased brain damage occurred in spite of the fact that the mean level of serum

bilirubin attained was only 8.0 mg% which was considerably less than that in the oxytetracycline group, which had only one case of kernicterus and a maximum mean serum bilirubin level of 16.4 mg%. *In vitro* experiments have suggested that the gantrisin acts by displacing the bilirubin from serum and tissue albumin and that the 'free' bilirubin is then able to get into the brain. Other drugs such as asprin also act in this way and may make the newborn infant more susceptable to kernicterus[14].

We have seen that jaundice in the new born is often due to a deficiency of glucuronyl transferase and one may perhaps wonder whether this enzyme deficiency occurs in other species than the human. This has been shown to be the case in a homozygous strain of Wistar rats, known as Gunn rats, which have serum unconjugated bilirubin levels of about 5 mg/100 ml (Table 2). This

Table 2. Glucuronide Excretion in Jaundiced and Normal Rats

WISTAR RATS	COMPOUND GIVEN	DOSE GIVEN per 100 gm	MODE OF ADMINISTRA- TION	URINARY EXCRETION OF GLUCURONIC ACID (% of dose administration) MEAN OR RANGE
Jaundiced	o-aminobenzoic acid	mg. 25	Oral	22.8
Non-jaundiced litter mates	o-aminobenzoic acid	25	Oral	70.1
Normal Controls	o-aminobenzoic acid	25	Oral	93.2
Jaundiced	Soduim o-aminobenzoate	100	Intraperitoneal	8.4—10.9
Non-jaundiced litter mates	Sodium o-aminobenzoate	100	Intraperitoneal	25.4
Jaundiced	Menthol	25	Oral	28—42
Non-jaundiced litter mates	Menthol	25	Oral	70—72

R. Schmid et al.: J. Clin. Invest. 37, 1123, 1958

hyperbilirubinemia occurs in spite of a normal liver histology. If an ordinary rat is given an oral dose of aminobenzoic acid then 93% of the administered aminobenzoic acid will be excreted as glucuronide in the urine. The same dose of aminobenzoic acid to one of these jaundiced rats, however, will result in only 23.8% being excreted as glucuronide, thus indicating the difficulty they have in excreting substances as glucuronides. It can be shown that there is a very definite lack of glucuronyl transferase in the liver of the animal
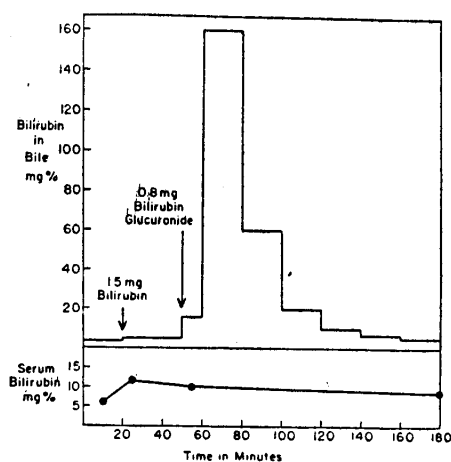
Fig. 8.   Bilirubin excretion (Schmid et al. :
J. Clin. Invest. 37, 1123, 1958).

and this is probably the reason that they remain jaundiced.

An elegant experiment by Dr. Schmid and his colleagues has shown that the excretory mechanism for conjugated bile pigments is normal (Fig. 8) in Gunn rats. He canulated the bile duct of one of these rats and then injected bilirubin, collected the bile and measured the concentration of bilirubin; little change was observed after the injection but if he gave bilirubin glucuronide instead of bilirubin then the increase in pigment concentration in the bile was very notable and equivalent to that observed in a normal rat given a similar does of bilirubin. In other words, the ability of the Gunn rat to excrete bilirubin glucuronide is normal. So that here is an example of jaundice in an animal which is due to an enzyme deficiency.

The possibility that a similar enzyme deficiency occurs in adults has been examined by studying four types of familial hxperbilirubinemia (Table 3). In the first group, originally described by Crigler and Najjar[15], the patients have serum bilirubin levels of the indirect type of about 20 mg/100 ml. and are often mentally defective, with brain damage associated with jaundice since birth. Some members of these families are, however, intensely jaundiced but otherwise normal. In these patients there is no doubt that the defect is an enzymic one, but in the less severe Gilbert's disease, in which the bilirubin level is probably not much more than 3 or 4 mg/100 ml, it is not possible to demonstrate so clearly that there is an enzyme defect. A more likely explanation for the jaundice seems to be a defect in the mechanism whereby bilirubin is transferred from the serum albumin into the liver cells. Exactly what the defect is I do not know, and this is only just a working hypothesis.

There are also two rather rare types of familial hyperbilirubinemia in which conjugated bilirubin, as well as bilirubin accumulates in the serum in spite of the absence of any evidence for an obstructive type of jaundice. The liver biopsy will be normal in the group of patients first described by Dubin and Johnson[16] except for a very curious black pigment in the centrilobular regions of the liver. This pigment is a very useful diagnostic aid but is not responsible for the ac-

Table 3.   Familial Hyperbilirubinemia

FAMILIAL HYPERBILIRUBINAEMIA				
	CRIGLER-NAJJAR	GILBERTS	DUBIN JOHNSON	ROTOR
Liver Histology	Normal	Normal	Pigmentation	Normal
Plasma Bilirubin	+ + +	+	· +	+
Conjugated Bilirubin	—	—	+ +	+ +
Defects Enzyme	+ + +	?	—	—
Bilirubin Uptake	+	+	?	?

Conjugated
    Bilirubin
    Secretion
	—	—	+ +	+ +

Bromsulphalein Retention	—	—	+ +	+ +

cumulation of conjugated pigment in the serum. In the other group, first described by Dr. Rotor and his group in the Philippines[17], the liver appears to be normal in structure when examined by normal light and electron microscopy so that same other reason must be found to explain why these people are jaundiced. We tried to investigate this matter by giving the patients an intravenous dose of bilirubin (2mg/kg). Such an administration in the normal results in an increase in the amount of indirect bilirubin in the serum and a very small increase in the amount of conjugated bilirubin; in four hours these levels have returned to normal but in the patient with the Rotor syndrome the picture is quite diffe-rent, for the indirect bilirubin concentration goes up and stays up and in four hours still has not returned to normal. The same thing happens with the con-jugated bilirubin thus indicating a defect in the excretion of conjugated bilirubin. This defect becomes more apparent in the case of the Dubin-Johnson syndrome illustrated in (Fig. 9). After the injection the total bile pigments in the serum rose quickly and then only very slowly decreased. The reason for this is not that the up-take of indirect bilirubin has almost completely stopped but that there is an increase in the conjugated bilirubin due to its reappearance in the circula-tion instead of the bile. It seems likely that this defect in excretion occurs at the
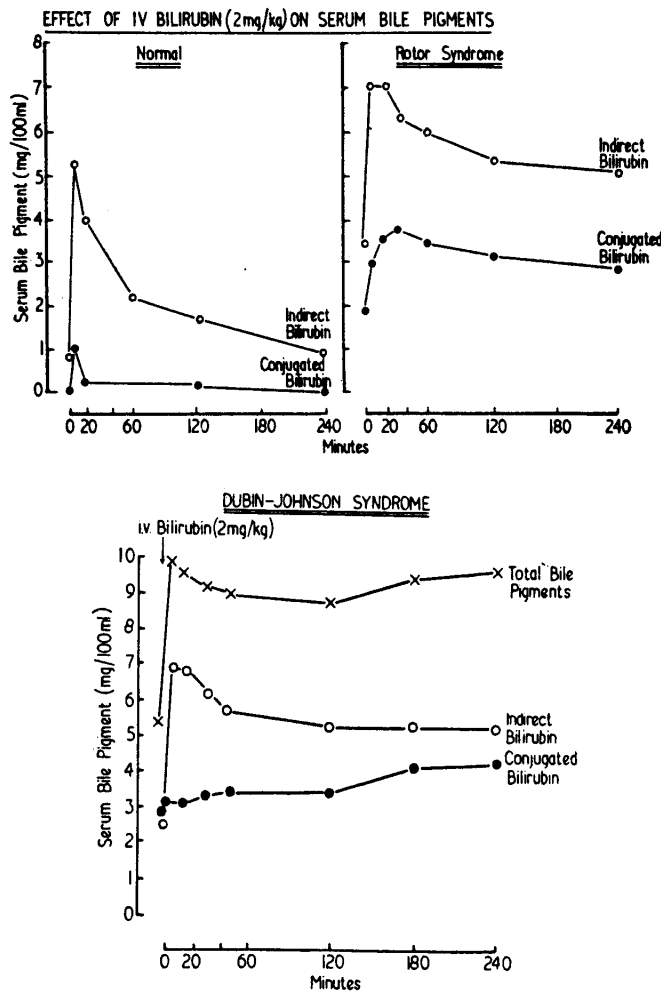
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Fig. 9. Effect of intravenous bilirubin (2 mg/Kg) on the serum bile
pigment concentration in a normal subject and patients with the Rotor
Syndrome and Dubin-Johnson Syndrome.

cell membrane but at present nothing is known of its nature. There is certainly
no doubt that the formation of glucuronide in these patients is perfectly normal.
This inability to excrete conjugated bilirubin is found on rare occasions in the
new born following exchange transfusions for erythroblastosis. 48 hours after
birth when sufficient glucuronyl transferase has developed it may be noted that
the indirect bilirubin level drops while the conjugated level rises so that the
figure for total bile pigment concentration remains relatively unchanged (Fig. 10).
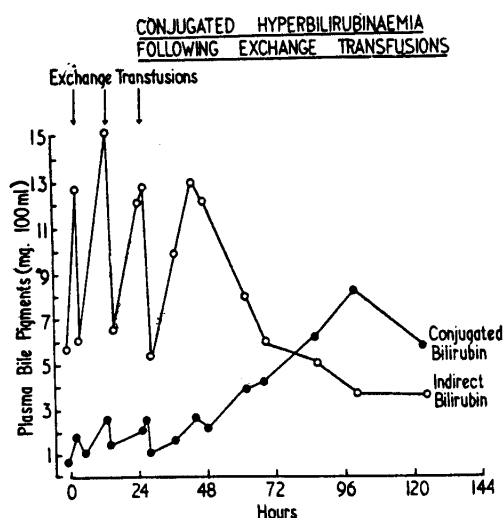
Fig. 10.   Conjugated hyperbilirubinemia following exchange
transfusions for erythroblastosis.

Dr. Jouvenceaux[18] in France has reported several families who have this tempo-
rary problem of excreting conjugated bilirubin.   In a week or so the jaundice
disappears.   Biopsies have not been performed but there is no clinical evidence
to suggest that any gross abnormality in the liver exists.

No mention so far has been made of renal excretion of bilirubin mainly
because not much is known about it.   From the literature it would appear that
bilirubin is excreted to a very small extent by the kidney and is a tubular pro-
cess.   It would have seemed reasonable to postulate that when one recovers from
jaundice that the disappearance of bile pigments could be accounted for by an
increased excretion of pigment in the urine or in the faeces.   However, as shown
in (Fig. 11) the urine bilirubin excretion parallels the fall in the serum level and
although there is an increase in the fecal urobilinogen this is considerably de-
layed and could not possibly explain the disappearance of the bile pigments.   So
we have the problem of what happens to the bile pigments when they disappear
upon release of an obstruction or recovery from hepatitis.   At present, I am
afraid we do not know the answer and this will obviously be another field for
considerable research.   Perhaps the pigment breaks down into dipyrrols, which
are colourless and do not give a positive van den Bergh reaction.   We are at
present in our laboratory making some radioactive bilirubin in the hopes that
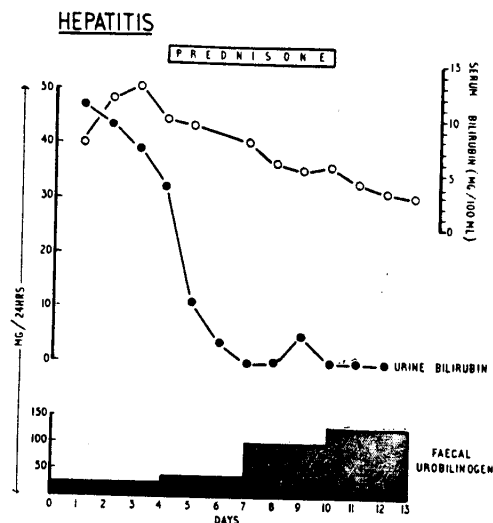this will give us some help with the problem.

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Fig. 11.   Changes in serum bilirubin, urine bilirubin and faecal uronilinogen in a patient with hepatitis treated with prednisone (50 mg. /day).

SUMMARY

The excretion of bilirubin and therefore the relief of jaundice is dependent upon at least three factors.   First, the bilirubin must be conjugated and thus converted into a water soluble compound: this means its conversion to an ester glucuronide although other conjugates may also be formed.   Secondly, there is the problem of the transport of bilirubin through the hepatic cell.   A defect in either the up-take of bilirubin or the secretion of conjugated bilirubin may result in jaundice such as is seen in the various types of familial hyperbilirubinemia.   Thirdly, there is the possibility of alternative catabolic pathways for bilirubin: this approach to the problem has, however, not yet received the attention of investigators.

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