BILIRUBIN METABOLISM

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DURING the last ten years considerable advances have been made in our knowledge of the metabolism of bilirubin and thus in our understanding of the basic defects underlying jaundice, particularly in the newborn and in some types of familial hyperbilirubinæmia. These advances are due mainly to the characterization of 'direct' bilirubin as an ester glucuronide of bilirubin. Many of the classical concepts of bilirubin metabolism can therefore now be explained in chemical terms.

An exciting new development has been the production of radioactive bilirubin (Ostrow, Hamaker and Schmid, 1961; Grodsky, Carbone, Fanska and Peng, 1962); this tool has enabled the existence of an entero-hepatic circulation for bilirubin to be demonstrated and will doubtless help unravel many of the unsolved problems of bilirubin metabolism.

Formation of bilirubin

Most of the circulating bilirubin results from the catabolism of hæmoglobin in the reticulo-endothelial system, particularly in the bone marrow, spleen and liver. The exact pathway of hæmoglobin breakdown first to biliverdin and then, after reduction, to bilirubin is still not clear. It is known that the administration of both hæmatin and protoporphyrin results in the formation of bilirubin but there is no conclusive evidence that either of these compounds is involved in the normal catabolism of hæmoglobin. *In vitro* studies have suggested that hæmoglobin may be degraded to bilirubin via choleglobin but its formation has not been demonstrated *in vivo*.

The studies of Gray, Neuberger and Sneath (1950), together with those of other investigators, have established that not all the bile pigment formed results from the breakdown of the red cells at the end of their life span. They used fæcal stercobilin as a measure of bile pigment production and after the administration of ^{15}N glycine determined its specific activity at suitable time intervals (Fig. 1). The ^{15}N stercobilin time-curve had two peaks; the first occurred immediately following the glycine feeding while the second had a maximum about the 130th day when the labelled red cells were breaking down. In the normal

FIG. 1.— ^{15}N contents of hæm in samples (full line) and of stercobilin (dotted line) at various times after administration of glycine (12 g.) containing 31.65% excess ^{15}N to a normal subject (Gray and others, 1950).

subject the first peak is responsible for approximately 10% of the pigment excreted but in diseases such as pernicious anæmia, congenital porphyria and thalassæmia 40-80% of the bile pigment excreted may be due to this fraction. It was originally thought that this 'early stercobilin' arose by direct synthesis from simple precursors such as aminolævulinic acid or porphobilinogen. It is now considered, however, to be mainly erythrohæmopoietic in origin since this early incorporation of labelled glycine into stercobilin is increased when erythrohæmopoiesis is stimulated. This erythrohæmopoietic stercobilin may be derived from three sources, namely, the immature red cells in the bone marrow (which are degrading hæmoglobin as well as synthesising it), from hæm formed in excess of globin or from hæm formed by the destruction of newly formed red cells as soon as they reach the peripheral circulation. A very small amount of bilirubin could theoretically come from hæm pigments such as myoglobin or the cytochromes but there is no information regarding the role played by these proteins in bile pigment formation. The possibility of an hepatic origin of the early labelled stercobilin has been suggested by the work of Watson-James and Abbott (1961) who found an increase in a patient with aplastic anæmia.

Bilirubin is a lipid-soluble, non-polar pigment which gives an 'indirect' reaction in the presence of alcohol in the van den Bergh test. Prior to excretion in the bile, it is converted into a pigment which is water-soluble at a physiological pH and gives a 'direct' van den Bergh reaction with diazotised sulphanilic acid. Using chromatographic techniques Cole and Lathe (1953) were able to show that the properties of 'direct' bilirubin were not due to a different type of protein attachment but to an alteration in the chemical structure of the pigment. By means of partition chromatography (Cole, Lathe and Billing, 1954) (Fig. 2) or paper chromatography (Giovannetti, Maggiore and Vivaldi, 1961) it is possible to demonstrate that 'direct' bilirubin consists of two components (pigments I and II). The most polar pigment (pigment II) is the main pigment present in bile and together with an intermediate pigment (pigment I) is found in the icteric urine and sera of patients with obstructive jaundice, hepatitis and cirrhosis. The extreme instability of these pigments has so far prevented their isolation in a pure form. The structure of pigment II has therefore been established by characterizing the azo-pigment formed in the van den Bergh reaction; this pigment (azo-pigment B) is considerably more polar than azo-pigment A and can be separated from it and other contaminants in diazotised bile by partition chromatography (Fig. 2), counter-current distribution (Billing, Cole and Lathe, 1957) and paper chromatography (Schmid, 1957). The application of the latter two techniques has shown azo-pigment B to be the ester glucuronide of azo-pigment A. Since two molecules of azo-pigment A are known to be formed from one molecule of bilirubin it has been concluded that pigment II behaves similarly and is therefore an ester diglucuronide of bilirubin, the glucuronic acid radicals being linked to the carboxyl groups of the propionic substituents of the molecule as indicated in Fig. 3. A similar conclusion was reached by Talafant (1956) as the result of experiments involving the electrophoresis of bile.

The structure of pigment I has been a more controversial matter. Since both azo-pigment A and azo-pigment B are formed from pigment I in the van den Bergh reaction it seemed likely that the pigment is the monoglucuronide of bilirubin. However, if pigment I is eluted from a chromatogram and then rechromatographed it has been observed that both bilirubin and pigment II appear on a second chromatogram (Billing, Cole and Lathe, 1957; Nosslin, 1960). This observation together with other studies (Gregory, 1962; Weber, Schalm and Witmans, 1963) gives support to the hypothesis that pigment I is a labile equimolecular complex of bilirubin and bilirubin diglu-

FIG. 2.—Behaviour of bile pigments and their diazo products on reverse phase partition chromatograms (Nosslin, 1960).

FIG. 3.—Structure of bile pigments (bilirubin, R_1 and $R_2 = H$; Pigment I, $R_1 = \text{glucuronyl}$ and $R_2 = H$; pigment II, R_1 and $R_2 = \text{glucuronyl}$; Me = methyl and V = vinyl).

curonide, and further investigations are now needed to determine the factors involved in its formation.

It is generally accepted that over 80% of the bilirubin in bile is normally present as the glucuronide. Examination of chromatograms and the observation that mild alkali treatment does not convert all the 'direct' bilirubin in icteric serum or bile into bilirubin indicate that other conjugates may be present. Isselbacher and McCarthy (1959) by means of $^{35}\text{SO}_4$ tracer studies have demonstrated the presence of sulphate conjugates of bilirubin in the bile of rats, cats and humans. These findings have been confirmed in the rat by Schoenfield, Bollman and Hoffman (1962) who demonstrated that the sulphate conjugate of bilirubin is associated with pigment II but not with pigment I. Gregory and Watson (1962) have not, however, been able to demonstrate the presence of bilirubin sulphate in the bile of dogs or human subjects and question the assumption of Isselbacher and McCarthy that alkali-stable 'direct' bilirubin is identical with bilirubin sulphate. At present there is no evidence that defective conjugation of bilirubin as a glucuronide

in man can be compensated for by an appreciable increase in the excretion of bilirubin as a sulphate or some other conjugate.

Biosynthesis of Bilirubin Glucuronide

It has been demonstrated that uridine diphosphate glucuronic acid (UDPGA), and not glucuronic acid, is the glucuronyl donor necessary for the formation of bilirubin glucuronide (Fig. 4). The conjugating enzyme is a glucuronyl transferase (UDP-trans-glucuronylase) which is located in the microsomes; the evidence regarding the specificity of this enzyme is equivocal. Both the kidney and gastrointestinal tract as well as the liver contain an enzyme system capable of conjugating bilirubin. In the intact adult animal no satisfactory evidence has, however, been presented to indicate that conjugation of bilirubin takes place in any organ other than the liver. Hepatectomy in experimental animals results in the gradual accumulation of both bilirubin and a pigment which gives a 'direct' van den Bergh reaction and behaves chromatographically like pigment I. This pigment has been identified in the dog as bilirubin monoglucuronide (Schoenfeld, Grindley, Foulk and Bollman, 1961) but the site of its formation under these abnormal conditions has not been established.

The ability of the fetal and newborn liver to form bilirubin glucuronide is markedly reduced, due mainly to a deficiency of glucuronyl transferase activity rather than UDPGA. In the rat fetus the glucuronyl transferase activity of the gastric mucosa is twice that of the adult liver (Stevenson and Dutton, 1962). The possibility that the gastric mucosa might be an important means of removing bilirubin in the fetus has been considered but seems unlikely in the light of recent studies using ^{14}C bilirubin in the guinea-pig fetus. (Schenker, Dawber and Schmid, 1962) and the monkey fetus (Lester, Behrman and Lucey, 1962), which showed that fetal bilirubin can be efficiently removed, after crossing the placenta, in the maternal bile.

Enterohepatic Circulation of Bilirubin

The experiments of Lester, Ostrow and Schmid (1961) in which ^{14}C bilirubin was administered intraduodenally to rats with bile fistulae have clearly demonstrated that in the rat, bilirubin is absorbed from the intestine and then reappears in the bile. The absorption of unconjugated bilirubin appeared to be more rapid and quantitatively greater than that of conjugated bilirubin. It seems likely that conjugated bilirubin is first hydrolyzed to bilirubin and then absorbed as free bilirubin.

The existence of an enterohepatic circulation

FIG. 4.—A possible mechanism for the conjugation of bilirubin with glucuronic acid (Billing and Lathe, 1958).

for bilirubin in man has been confirmed using both bilirubin labelled with ^{14}C (Lester and Schmid, 1962) and bilirubin labelled with ^{15}N (Gilbertsen, Bossenmaier and Cardinal, 1962). The significance of the enterohepatic circulation in normal man remains to be investigated. It may provide one of the reasons for the lack of correlation between the amount of pigment known to be formed from the breakdown of circulating red cells and that found as stercobilin in the faeces. In patients with a compensated hyperbilirubinemia due to a deficiency of glucuronyl transferase (Crigler-Najjar syndrome) and a homozygous strain of rats (Gunn rats) with the same deficiency, Schmid and Hammaker (1962) have found that unconjugated bilirubin can be both absorbed by the intestine and transferred from the plasma pool directly across the mucosa into the intestinal lumen. In the Gunn rats they were able to prevent its reabsorption by feeding cholestyramine resin, which has a high affinity for bilirubin, and in this way significantly reduced the serum bilirubin levels to 30 to 45% of the control values (Lester, Hammaker and Schmid, 1962). The value of cholestyramine treatment in reducing jaundice in man remains to be assessed.

Conversion of Bilirubin to Urobilinogen and Mesobilifuscin

It has been well established that bilirubin, especially if in the conjugated form, is reduced

by the bacterial flora in the intestine and colon to the urobilinogen group of pigments, which give the well-known Erlich's aldehyde reaction. These urobilinogens are readily dehydrogenated to give urobilin IX α , stercobilin and d-urobilin, the relative amounts formed depending on the mobility of the intestinal contents and the rate of filling of the colon, as well as the efficiency of the bacterial flora. Watson and Weimer (1959) were unable to correlate the composition of the faecal urobilin with the patient's condition and while urobilin IX α was the dominant pigment in some subjects, stercobilin predominated in others. The administration of broad-spectrum antibiotics may result in bilirubin rather than stercobilin being excreted in the faeces.

The presence of an enterohepatic circulation for urobilinogen was first postulated by McMaster and Elman (1927). They assumed that in liver disease re-excretion of urobilinogen into the bile was impaired and that instead, the pigment passed to the kidney and was excreted in the urine. Urobilinoid pigments are, however, not normally found in bile except in the presence of infection; if McMaster and Elman's hypothesis is correct this means that the pigment would have to be reconverted to bilirubin in the liver before it could be excreted in the bile which, for biochemical and structural reasons, seems unlikely. A critical re-examination of this problem with labelled stercobilin of high specific activity is required to establish whether urobilinogen, like bilirubin, after absorption from the intestine is re-excreted in the bile. Recent experiments by Kahan, Csernay and Varro (1962) have shown that the reabsorption of stercobilin from the isolated small intestine of the dog is dependent on the formation of a mucoprotein complex and suggest that the kidney may play some part in the conversion of urobilin to its respective urobilinogen prior to excretion in the urine.

It has been suggested that the discrepancies observed between urobilinogen excretion and haemoglobin catabolism might be accounted for by the conversion of bilirubin or urobilinogen to dipyrromethenes such as mesobilifuscin. Studies by Gilbertsen and Watson (1962) support the view, however, that the naturally occurring fecal dipyrromethene pigments are anabolic rather than catabolic in origin.

Bile Pigments in Plasma, Urine and Body Fluids

At the serum bilirubin levels encountered in icteric plasma, regardless of the cause of the jaundice, virtually all the pigment is bound to albumin (Ostrow and Schmid, 1962). It is for this reason that attempts to reduce jaundice by

dialysis have been for the most part unsuccessful. If however albumin is added to the dialysis fluid then it is possible for considerable quantities of bilirubin to be removed by intermittent peritoneal dialysis (Grollman and Odell, 1962).

Conjugated bilirubin is also bound to the plasma albumin but the type of pigment-protein-complex formed is probably different (Klatskin and Bungards, 1956). It may also form a complex with phospholipids which can be extracted by shaking with ether (Charbonnier and Pougouras, 1959; Lucassen, 1961). This 'ether-soluble bilirubin' extraction test has been used to differentiate between malignant and non-malignant biliary obstruction but according to Mertens and Croal (1960) is probably merely a measure of the degree of obstruction, as indicated by the high phospholipid level.

Small doses of bilirubin are quickly cleared from the plasma by the liver and then after storage and concentration are excreted into the bile at a slower rate. In the normal subject serum levels greater than 1 mg/100 ml are rarely encountered and the bilirubin is almost entirely in the non-conjugated form. With raised levels of serum bile pigments, bilirubin will be stored in most of the tissues in the body, including the adipose tissue. It has been suggested that the staining of the tissues of the skin and muscle may be associated with their extravascular albumin content (Billing and Lathe, 1958). The differences in skin colour observed in infants with haemolytic jaundice and adults with obstructive jaundice could be due to differences in tissue binding of bilirubin and conjugated bilirubin but these matters need to be investigated further.

Both unconjugated and conjugated bilirubin may be found in the lymph, ascitic fluid, pleural fluid and cerebrospinal fluid of the jaundiced subject. No correlation has been found between the bile pigment levels in the CSF and those in the plasma.

Bilirubin cannot usually be detected in the urine of normal subjects, or patients with an unconjugated hyperbilirubinemia. In icteric urine both pigments I and II but not bilirubin are found. The level of conjugated bilirubin in the urine does not correlate well with that in the plasma and depends on the state of the disease process. In viral hepatitis, for example, bile pigments may appear in the urine even before the patient becomes clinically icteric but during convalescence no pigment will be found in the urine although the serum level may be as high as 6-8 mg/100 ml. Lack of pure specimens of conjugated bilirubin and suitable techniques for the quantitative determination of bile pigments in urine has prevented satisfactory renal clearance

studies being carried out. It would appear that in the dog the pigments are excreted by the renal tubules, but there is no reliable evidence in man on this matter.

Toxicity of Bilirubin and Kernicterus

The yellow pigment that accumulates in the brain in kernicterus has been identified as unconjugated bilirubin. The toxic effects of bilirubin have therefore been studied and it has been shown that bilirubin has an inhibitory action on the oxygen consumption of brain tissue, particularly in the newborn. It has also been demonstrated that bilirubin is an inhibitor of hæm synthesis and that in rat liver and brain mitochondria it uncouples oxidative phosphorylation. According to Ernster (1961) bilirubin behaves like a detergent in its action on mitochondria and causes swelling as well as inducing ATPase activity. With high concentrations of bilirubin the mitochondria are destroyed, and an enzyme may be released which will oxidise bilirubin to biliverdin. It is possible that such a reaction may be responsible for the presence of small amounts of biliverdin in the serum of patients with advanced liver disease.

The newborn animal is more susceptible to the toxic action of large doses of bilirubin than the adult animal (Rozdilsky, 1961). In some animals staining of the nervous tissue is only observed if there is previous brain damage by severe hypoglycæmia, anoxia or traumatic hæmorrhage. Whether previous damage as well as high concentrations of bilirubin are necessary for the development of kernicterus in man has not been established. Neither is it known why some parts of the brain (e.g. the corpora striata, the thalamus and the hippocampus) tend to become stained in the presence of high levels of plasma bilirubin while others do not. The part played by the blood-brain barrier has also still to be clarified. There is suggestive evidence that in the newborn the permeability is increased so that bilirubin passes more readily into the CSF and the brain.

Conjugated bilirubin, on the other hand, appears to be non-toxic. In the adult with severe jaundice these pigments are predominant and only on rare occasions are high levels of unconjugated bilirubin encountered. This is probably the main reason for the apparent absence of kernicterus in the adult.

JAUNDICE

On theoretical grounds increased concentrations of bilirubin in the plasma may result from one or more of the following causes (Fig. 5).

(1) An increased load of bilirubin.

FIG. 5.—Jaundice could theoretically result from (1) an increased load of bilirubin; (2) defective uptake and transport within the liver cell; (3) defective conjugation in the hepatic microsomes; (4) defective canalicular excretion or a mechanical block in the bile duct system. (S. Sherlock (1962), *Brit. med. J.*, i, 1950).

(2) Defective uptake and transport within the liver cell.

(3) Defective conjugation of bilirubin in the hepatic microsomes.

(4) Defective canalicular excretion or mechanical block in the bile duct.

Although, in most instances, jaundice is not due to a single causative factor this may be the case in congenital hyperbilirubinæmia. Detailed studies of bile pigment metabolism in these disorders may, therefore, give some insight into the pathways of normal metabolism.

1. Increased load of bilirubin

Hæmolytic disease

An excessive release of hæmoglobin from the red cells may result in the normal daily production of 300 mg of bilirubin being increased as much as six-fold. The capacity of the liver to excrete bilirubin is, however, greatly in excess of that normally required so that in hæmolytic disease the plasma bile pigment concentration rarely rises above 5 mg/100 ml. The main pigment in the plasma is unconjugated bilirubin so that bilirubinuria is not usually observed. Small amounts of conjugated bilirubin may be detected in the plasma but these will not exceed 15% of the total serum bilirubin concentration unless hepatocellular damage has also occurred (Tisdale, Klatskin and Kinsella, 1959).

Alternative pathways of bilirubin metabolism

An overproduction of bilirubin can arise from sources other than the breakdown of mature red cells. Israels, Suderman and Ritzman (1959)

recently suggested that such a mechanism might be responsible for the unconjugated hyperbilirubinæmia they observed in four cases of jaundice, who had raised values of faecal and urine urobilinogen excretion, in the presence of a normoblastic hyperplasia of the marrow. Determinations of the relative specific activities of hæm and stercobilin following the intravenous administration of 50 microcuries of 2-¹⁴C-glycine to one of these patients, who had an average daily excretion of 945 mg stercobilin, indicated that at least 82% of this pigment was derived from sources other than the circulating red cells. Israels and Zipursky (1962) consider that this excess formation of bilirubin probably occurs in the bone marrow, either as a result of hæm catabolism or by a more direct anabolic pathway. They have called this 'shunt hyperbilirubinæmia' and consider that it represents a marked exaggeration of a normal pathway which is responsible for approximately 11% of bile pigment production.

Excessive formation of bile pigments in the presence of minimal peripheral hæmolysis has also been cited by Arias (1962a) and Robinson, Vanier, Desforbes and Schmid (1962) as a cause of chronic jaundice. The latter authors in their studies of the kinetics of bile pigment formation in a patient with thalassæmia minor, in whom there was marked evidence of 'ineffective erythropoiesis', were able to account for the pattern of stercobilin labelling by the greatly increased turnover of hæmoglobin in the bone marrow.

2. Defects in hepatic uptake of bilirubin

Almost nothing is known of the mechanism whereby bilirubin is transferred from the plasma via the sinusoids into the liver cell where it is actively transported to the microsomes for conjugation. A defect at some stage in this transport mechanism has been shown to occur in patients with 'Gilbert's Disease'. These comprise a heterogeneous group of benign disorders characterized by a mild unconjugated hyperbilirubinæmia of 1-4 mg/100 ml (Billing and Williams, 1962). Hepatic function tests are normal, except for an impaired bilirubin tolerance, and the composition of the bile and the faecal urobilinogen excretion are also normal. Liver histology shows only minimal changes. Very occasional reports of reduced ⁵¹Cr red blood cell survival times have been made in the presence of a normal reticulocyte count and a normal hæmoglobin concentration (Foulk, Butt, Owen and Whitcomb, 1959; Arias, 1962b) but there is no evidence of overt hæmolysis. No satisfactory evidence has so far been obtained which demonstrates a deficiency of glucuronyl transferase in these patients with Gilbert's disease. In the rarer type of patient with a plasma

level greater than 8 mg/100 ml it seems likely that the jaundice can be partly explained by an enzyme defect the extent of which appears to be related to the degree of jaundice.

3. Defects in bilirubin conjugation

Jaundice in the Newborn

Hepatic immaturity. In the newborn infant, unconjugated bilirubin will accumulate in the plasma until the necessary enzyme systems have developed to enable conjugation and excretion of the pigment to take place. It has been claimed that over 40% of newborn infants develop clinical jaundice of varying degrees of severity (Claireaux 1960). This is most likely to occur in premature infants who attain a higher degree of jaundice and remain jaundiced for a longer time than those with normal birth weights. It is only on rare occasions that the plasma bilirubin concentration exceeds 20 mg/100 ml, and, according to current practice, the need for an exchange transfusion has to be considered. This involves the removal of bilirubin not only from the plasma but also from the tissues. The administration of albumin prior to an exchange transfusion has therefore been suggested (Odell, 1959), in order to obtain maximum transference of bilirubin into the vascular compartment. Although the procedure has still to receive clinical evaluation, results obtained from experimental work with puppies (Waters, 1961) would support the use of albumin as a therapeutic agent and encourage the use of whole blood rather than packed cells. Preliminary reports proposing that glucuronic acid might be useful in preventing kernicterus has not been substantiated: on theoretical grounds this is not surprising since it is UDPGA and not glucuronic acid which is the glucuronyl donor for the synthesis of bilirubin glucuronide.

It has been known for some time that bilirubin is readily destroyed by light and Cremer, Perryman and Richards (1958) and Ferreira, Cardim and Mellone (1960) have claimed that exposure of newborn infants to artificial light is a useful procedure for the treatment of jaundice of the newborn. The *in vitro* studies of Blondheim, Lathrop and Zabriskie (1962) suggest that the light treatment results in the formation of water-soluble derivatives which are not bound by albumin. Whether these derivatives have any deleterious effect on the infant needs to be investigated and proper control studies are necessary before this form of treatment can be generally accepted as advantageous.

It is difficult to assess the extent to which jaundice in the newborn constitutes a pathological condition or may be described as 'physiological jaundice'. In the following discussion of other

factors influencing the development of jaundice in the newborn it is necessary to remember that there may be a pre-existing defect in conjugation and the concentration of unconjugated bilirubin in the plasma must be interpreted accordingly.

Hæmolytic disease. In hæmolytic disease, whether due to ABO or Rh incompatibility, the dominant bile pigment in the plasma is bilirubin. This tends to rise more quickly and to greater heights in the premature infant, since in addition to hepatic immaturity the infant has to contend with a greatly increased production of bilirubin due to excessive hæmolysis. The jaundice accordingly lasts longer.

The plasma of infants with hæmolytic disease very occasionally contains conjugated bilirubin in the cord blood and in subsequent blood samples. If this develops within 24 hours of birth it is usually associated with severe anæmia and hepatosplenomegaly, and the presence of high values for serum aspartate transaminase suggests hepatocellular damage. Harris, Farrell, Shorter, Banner and Mathieson (1962) in their post mortem study of the liver histology of eight such infants could find no evidence of obstruction of the bile canaliculi by islands of extramedullary hæmopoiesis or swollen hepatic cells, regurgitation of bile by damaged liver cells or inspissation of viscous bile. The term 'inspissated bile syndrome' would therefore appear to have no real meaning in this context (Brent, 1962) and, as will be discussed later, the accumulation of conjugated bilirubin is probably due to a secretory defect. Since conjugated bilirubin does not appear to be toxic the serum level of unconjugated bilirubin rather than of total bilirubin should be the governing factor in assessing the need for an exchange transfusion in these patients.

Glucose-6-phosphate-dehydrogenase deficiency. An hereditary deficiency of the enzyme glucose-6-phosphate dehydrogenase in the red cell can render it susceptible to hæmolysis by various agents including drugs and fava beans. This genetic defect may become apparent in the newborn reactor following the administration of sulfanilamide, naphthalene, primaquine or nitrofurantoin. Maternal medication with these drugs can result in this response occurring in the infant *in utero*.

Recent reports from Singapore (Smith and Vella, 1960), Greece (Doxiadis, Fessas and Valaes, 1961) and Sardinia (Panizon, 1960) have indicated that in certain parts of the world a deficiency of glucose-6-phosphate dehydrogenase may be an important factor in the ætiology of severe neonatal jaundice in mature infants, in whom no blood group incompatibility has been

recognized. Some of the jaundiced female infants, although non-reactors themselves, have been found to have fathers who are reactors. The severity of the jaundice, therefore, does not appear to be solely dependent on the degree of the enzyme defect. Fessas, Doxiadis and Valaes (1962) have found that in certain families the incidence of jaundice was greater than that found in 786 randomly selected Greek male neonates (2.92%), which suggested that there are probably additional genetic factors contributing to the development of severe jaundice. The jaundice disappears within a month of birth and does not reappear unless a suitable challenge is presented, such as the ingestion of fava beans; in this way the disease can be differentiated from congenital non-spherocytic hæmolytic anæmia. This condition appears to be an extremely unlikely cause of neonatal hyperbilirubinæmia in non-Mediterranean countries in Europe.

Vitamin K. It is generally accepted that a single dose of 2 to 5 mg. of a water-soluble vitamin K analogue is effective in preventing hæmorrhagic disease in the newborn and does not cause hyperbilirubinæmia. If, however, the dosage is increased then the likelihood of kernicterus developing is also increased (Bound and Telfer, 1956). The toxic effect is apparent in menadiol sodium diphosphate (Synkavit) vitamin K₁ and some of the other analogues, but not in menaphthone dipotassium sulphate (Vikastab) (Corner, Berry and Neale, 1960). The mechanism whereby vitamin K analogues cause jaundice is not well understood. Vest (1958) has shown that the erythrocytes of premature infants given vitamin K, in large therapeutic doses, have a shortened survival and *in vitro* studies with neonatal erythrocytes incubated with Synkavit have shown a rapid decrease in the reduced glutathione content, possibly due to interference in the regeneration of TPNH (Broberger, Ernster and Zetterström, 1960).

Non-hæmolytic Factors. Lathe and Walker (1958) have demonstrated that in the later stages of pregnancy the plasma of the mother contains an unknown substance which will inhibit conjugation of bilirubin by rat-liver slices. Whether this substance normally crosses the placenta into the foetal circulation and if so what its action *in vivo* would be, is unknown. It is, however, of interest that studies in infants with severe 'Transient Familial Hyperbilirubinæmia' (Lucey and Driscoll, 1961) showed that the plasma of these infants and their mothers has this inhibitory action on *in vitro* conjugation of bilirubin. The relationship of the inhibitor substance to the pathogenesis of the

jaundice, which subsided within the first month of life has, however, not been established.

In a clinical trial of antibiotics in premature infants it was observed that the administration of sulfisoxazole reduced the concentration of plasma bilirubin and at the same time increased the incidence of kernicterus (Harris, Lucey and Maclean, 1958). Experiments with *in vitro* systems and genetically jaundiced Gunn rats (Johnson, Garcia, Figueros and Sarmiento, 1961) have shown that organic anions such as sulphonamides and salicylates compete with bilirubin for albumin binding sites. An inverse relationship exists between the blood level of the drug and the plasma bilirubin. The distribution of bilirubin in the body is therefore altered and there is an increase in the amount of pigment entering the brain which may cause kernicterus. These investigations emphasize the impossibility of assessing the success of a therapeutic agent in reducing jaundice in the newborn merely by determining the serum bilirubin level; evidence of an increased excretion of bilirubin must be obtained.

Another drug known to cause jaundice is novobiocin (Cox, Foltz, Raymond and Drewyer, 1959); in the newborn it has been reported as causing a threefold increase in neonatal hyperbilirubinemia (Sutherland and Keller, 1961). *In vitro* studies (Hargreaves and Holton, 1962) suggest that novobiocin, which is excreted as a glucuronide, acts by competing with bilirubin for the very limited amount of glucuronyl transferase present in the newborn liver.

Jaundice in the Adult

Crigler-Najjar Syndrome (Congenital non-haemolytic jaundice). In very rare instances the deficiency of glucuronyl transferase seen in the newborn may continue into adult life. This syndrome was first described by Crigler and Najjar (1952) who studied the children of three related families with serum levels of unconjugated bilirubin ranging from 12-45 mg/100 ml. Brain damage is often caused in the neonatal period so that many of these patients, if they survive, are to be found in mental hospitals. However, if neurological symptoms do not develop during early life then the prognosis is probably good (Childs and Najjar, 1956; Sugar, 1961) since the toxic effects of bilirubin are less marked in the adult. These patients have an impairment in their ability to form glucuronides with aglycones such as salicylates, n-acetyl p-amino phenol, menthol and tetrahydrocortisone (Schmid, 1960). Only trace amounts of bile pigments are found in the bile and faecal urobilinogen excretion is low. *In vitro* studies have shown that there is a deficiency

of hepatic glucuronyl transferase but not of UDPGA, which is responsible for their jaundice (Jervis, 1959; Szabo, Kovacs and Ebrey, 1962). In all other respects liver function appears to be normal and there is no evidence of haemolytic disease.

The serum bilirubin levels remain relatively constant in these patients in spite of a normal rate of haemoglobin breakdown and defective pigment excretion. Some alternative pathway for the removal of bilirubin must therefore be present which, although less efficient than the usual conjugating system since hyperbilirubinemia occurs, is capable of maintaining a 'steady state' with respect to bilirubin. Schmid and Hammaker (1962) have used ^{14}C bilirubin to estimate the rate of turnover and the magnitude of the total miscible pool of bilirubin in a young boy with congenital non-haemolytic jaundice. Most of the isotopic bilirubin appeared in the faeces in the form of metabolites exhibiting properties different from those of the known bile pigments. Further work is needed to establish the site of breakdown of the bilirubin and the nature of the metabolites.

4. Disturbances in hepatic excretion of bilirubin

Obstructive jaundice and hepatocellular disease

Since over 90% of the pigments in hepatic bile occur as pigment II it would be expected that this would be the dominant pigment in the plasma of patients with extrahepatic biliary obstruction. If however the obstruction continues for some weeks then the proportion of pigment II in the serum will decline while that of pigment I will increase. A similar pigment pattern is then obtained to that found in the plasma of patients with parenchymal liver disease due to hepatitis or cirrhosis which has more pigment I than pigment II or bilirubin (Billing and Lathe, 1958). Hoffman, Whitcomb, Butt and Bollman (1960) examined the pigment patterns of 150 cases of jaundice and endeavoured to use them to differentiate between acute obstructive jaundice and hepatocellular disease. There was unfortunately a significant overlap between the two groups which prevents the procedure from being a useful diagnostic tool; it is not helpful in distinguishing between extra and intra-hepatic cholestasis.

Treatment with ACTH and corticosteroids has been used for the differential diagnosis of chronic jaundice with some degree of success. In infective hepatitis there is usually an immediate clinical improvement accompanied by a sharp decline in the plasma bilirubin level, which cannot be explained in terms of increased biliary excretion or a change in the renal excretion of bile pigments; the rate of red cell breakdown is also unchanged

(Williams and Billing, 1961). In cases of intra- and extra-hepatic obstructive jaundice and drug jaundice a favourable response is rarely seen. No satisfactory explanation has so far been put forward for the altered bile pigment metabolism following steroid therapy in hepatitis.

Drug Jaundice

The first report of drug jaundice was by Hanger and Gutman (1940) in which 12 patients receiving intravenous arsphenamine for the treatment of syphilis became icteric after the second or third injection. Since then an ever-increasing number of drug reactions with jaundice due to intra-hepatic cholestasis has been described (Popper and Schaffner, 1959). With many of these drugs such as chlorpromazine ('Largactil'), thiouracil, para-aminosalicylic acid, chlorpropamide and cetyl urea, the jaundice has been attributed to a hypersensitivity reaction. 1% of patients receiving chlorpromazine treatment develop frank jaundice together with increases in serum alkaline phosphatase and transaminases and also bromsulphthalein retention. Out of eight patients studied by Hoffman and others (1960) four had a pigment pattern similar to that seen with extra-hepatic obstruction, while the other four patients had a pattern suggestive of hepatocellular damage.

Anabolic agents, such as methyl testosterone and norethandrolone ('Nilevar') with a C17 alkyl substitution in the steroid molecule, may cause jaundice to appear late in the course of therapy, without symptoms of a hypersensitivity reaction. Abnormalities in the microvilli lining the biliary canaliculi have been reported following the administration of these drugs and it is thought that these may be responsible for the bile stasis (Schaffner, Popper and Perez, 1960). Experimental work with rats also supports the view that norethandrolone acts by interfering with the excretory function of the liver cell.

Another group of drugs occasionally causes a very severe type of jaundice, which is histologically and biochemically indistinguishable from viral hepatitis. This type of liver injury may follow the administration of the mono-amine oxidase inhibitors iproniazid ('Marsilid'), pheniprazine, β -phenyl isopropyl hydrazine, isocarboxazid ('Marplan') and phenazine ('Nardil'). It has also been reported with iso-nicotinic acid, hydrazine, pyrazinamide, zoxazolamine and the oral hypoglycaemic agents metahexamide and tolbutamide.

Other drugs may cause jaundice as a result of direct hepatocellular damage, which affects both the conjugation and excretion of bilirubin. Drugs in this group include carbon tetrachloride, chloroform, ethyl chloride, dichloro-diphenyl-trichloroethane (D.D.T.) as well as muscarine,

FIG. 6.—Effect of intravenous bilirubin (2 mg./kg.) on the serum bile pigment concentration of a patient with the Dubin-Johnson syndrome.

metallic poisons, naphthalene and benzene derivatives.

Functional defects in excretion

Defects in the excretory function of the liver need not necessarily be accompanied by any gross histological abnormality of the bile ducts. The accumulation of conjugated as well as unconjugated bilirubin suggests that the difficulty lies between the microsomes and the bile canaliculi. It appears to be developmental in origin but further investigations of the mechanism of pigment excretion are necessary to establish whether the defect is a structural or metabolic one.

It has been observed that in some infants with haemolytic disease that the fall in level of plasma unconjugated bilirubin is preceded by a rise in the conjugated pigment concentration. This indicates that the conjugating enzyme system has developed satisfactorily but that the secretory function of the liver cell is still inadequate (Harris and others, 1962). This impairment in excretion may occasionally persist for several weeks and some patients appears to have a familial incidence (Jouvençaux, Brizard, Michaud and Revol, 1954; Billing, 1961).

In some cases of chronic non-haemolytic jaundice in adolescents and adults conjugated as well as unconjugated bilirubin is present in the

plasma in spite of the absence of any histological evidence of an obstructive type of jaundice or hepatocellular lesion. When these patients are given an intravenous injection of bilirubin there is a rise in the plasma concentration of conjugated bilirubin at the time that the level of unconjugated bilirubin is falling, indicating a defect in hepatic excretion (Fig. 6). This excretory defect is not limited to bilirubin since there is retention of bromsulphthalein and other dyes after an intravenous dose. These patients also have difficulty in excreting cholecystographic contrast media. Other liver function tests are usually normal.

The patients can be divided into two groups dependent on the presence or absence of a yellow-brown granular pigment in the centrilobular regions, which give the liver a macroscopic black

appearance. It is not thought that the pigment bears any relationship to the presence or intensity of the hyperbilirubinemia (Wolf, Pizette, Richman, Dreiling, Jacobs, Fernandez and Popper, 1960). The patients with the lipochrome pigment are said to have the Dubin-Johnson syndrome (Dubin, 1958) while those without have the less common Rotor syndrome (Rotor, Manahan and Florentin, 1948; Schiff, Billing and Oikawa, 1959). An interrelationship appears to exist between the two syndromes since in a particular family some affected members may have the pigment, while others who are also jaundiced do not (Arias, 1961). Elucidation of the pathogenesis of these genetically determined disorders should give valuable information about the normal mechanism of excretion by the liver cell.

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