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THE FORMATION OF BILIRUBIN MONO- AND DIGLUCURONIDE AND p-NITROPHENYL GLUCURONIDE

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. MR. F.J.F.M. DUYNSTEE VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP VRIJDAG 21 FEBRUARI 1975, DES NAMIDDAGS TE 2.00 UUR PRECIES.

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CONTENTS

INTRODUCTION

Lipophilic drugs, food additives, and environmental pollutants, that enter the body via ingestion, inhalation, or penetration through the skin, have a tendency to accumulate in the fatty tissues or to remain bound to certain proteins. They can only be cleared from the body after a more or less extensive alteration of their chemical structure. The reactions leading to these alterations are catalyzed by the biotransformation enzymes, which are predominantly localized in the liver.

Oxidations and conjugations are the main types of biotransformation processes. The oxidative metabolism of drugs, steroids and fatty acids is mediated by an enzyme system in the endoplasmic reticulum of the liver, which consists of a CO-binding hemoprotein (cytochrome P-450), NADPH-cytochrome P-450 reductase and lipid[1,2].
The hemoprotein and the flavoprotein, cytochrome P-450 reductase, have been partially purified[3]. Study of this enzyme system is facilitated by the fact that the spectrum of the cytochrome, and changes therein through ligand binding or induction, can be directly measured. Through oxidation many compounds groups to which conjugating moieties can be easily attached. Oxidations and conjugations are therefore called phase-I and phase-II reactions, respectively.

Conjugation with sugar groups (in particular glucuronic acid), glycine, taurine, glutathione and sulfate are the most important phase II reactions. Conjugations of the first type are the subject of this thesis and will be discussed below.

A wellknown example for conjugation with glycine is the formation of hippuric acid from benzoic acid.
Glycine and taurine conjugation are important for the metabolism of bile acids. Enzymically these are interesting reactions. In a first, ATP-requiring step, the substrate reacts with CoA-SH to form a substrate-S-CoA complex. This step is catalyzed by a microsomal enzyme. In a second step the substrate is transferred from the intermediate complex to glycine or taurine and this step is probably catalyzed by a lysosomal enzyme[4].
Glutathione conjugation plays an important role by the detoxification of alkylating agents.
The glutathione S-transferases catalyzes the conjugation of glutathione with a number of compounds bearing an electrophilic center. They have been subdivided in aryl-, alkyl-, aralkyl and epoxide-transferases[5] but this nomenclature has recently been challenged. Four different enzymes with overlapping substrate specificities have been purified from rat liver cytosol[6,7]. Glutathione conjugation is the initial step by the formation of the mercapturic acids[5].

Phenols, steroids and tyrosine can form sulfate conjugates. Catalyzed by a sulfotransferase the sulfate group of 3'-phosphoadenylsulfate is transferred to an accepter substrate. There are indications that a whole family of sulfotransferases exists. Some of them have been partially purified from liver cytosol[8].

Besides conjugating enzymes, the body contains numerous hydrolases. This allows the possibility of a fine regulation of the metabolic processes. Some of the hydrolases, e.g. sulfohydrolases[9] and glucuronidases[10], may catalyze transconjugations. The hydrolytic enzymes are stored in the lysosomes but also the endoplasmic reticulum contains sulfohydrolases[9] and β-glucuronidases[11].

UDPglucuronic acid is formed by oxidation from UDPglucose[12]. UDPglucose dehydrogenase[12], a cytoplasmic enzyme, catalyzes the two oxidation steps which are required to convert the hydroxyl group of a primary alcohol into a carboxyl group. The intermediate of this reaction has not been detected but UDP-diglucoaldose is the most likely candidate[13]. The overall reaction requires two molecules of NAD[10,13]. UDPglucose, which is also the main precursor of glycogen synthesis, is formed from α-D-glucose-1-phosphate and UTP by a pyrophosphatase [10,13].

Besides the activity of UDPglucuronyltransferase, the possibility of glucuronide formation in vivo depends on the availability of the donor nucleotide. The concentration of UDPglucuronic acid in the cell is determined by the availability of UDPglucose, the activity of UDPglucose dehydrogenase, the utilization of UDPglucuronic acid in conjugation reactions, and its breakdown by pyrophosphatases and phosphatases. It has, for example, been shown, with liver homogenates of rats with experimentally induced diabetes through insulin deficiency, that the glucuronide formation was impaired whereby a decreased UDPglucose dehydrogenase activity was probably the rate limiting factor[14].

Decarboxylation of UDPglucuronic acid leads to the formation of UDP-xylose, which is an allosteric regulator of the oxidation of UDPglucose to UDPglucuronic acid[10,13]. UDPglucuronic acid, UDPxylose, UDP-N-acetyl glucosamine, UDP-N-acetyl galactosamine and UDPgalactose are important by the formation of mucopolysaccharides whereby they serve as donor substrates for donation of the sugar moieties[13]. In recent reports attention has been paid to the conjugation of small molecular compounds with sugars other than glucuronic acid and formation of glucuronic acid and formation of gluco-

sides[15,16,17], xylosides[15], galactosides[18] galacturonides[18a] and conjugates with N-acetylglucosamine[19] have been reported.

Glucuronide formation

The formation of β-D-glucosiduronates, or glucuronides, occurs by transference of the glucuronyl group of uridine 5'-pyrophosphate-D-glucopyranosiduronic acid to a suitable aglycone. Only UDPglucuronic acid with an internal α-link can serve as donor of the sugar group[20,21]. During formation of glucuronides an inversion of this link occurs as β-glucuronides are formed. It has been postulated that this can occur by nucleophilic substitution in which the electron donating aglycon displaces UDP from the opposite side of the electrophilic anomeric C-atom of UDPglucuronic acid[22].

Glucuronic acid and glucuronic acid 1-phosphate cannot serve as donor substrates, nor can glucuronolactone[10]. Only certain N-glucuronides can be formed in the presence of glucuronic acid, but this occurs non-enzymically[10]. It has been postulated that, in vitro, glucuronyl transfer can occur from the glucuronides of e.g. stilbestrol, phenol, menthol and phenolphthalein to alcohols. The reaction occurs in the presence of a β-glucuronidase-like enzyme[23]. These findings however still await rigorous confirmation.
The glucuronidation of p-nitrophenol is a reversible reaction[24]. This opens the possibility of glucuronyl transfer from a glucuronide to an aglycone, with UDPglucuronic acid as an intermediate[10].
Recently, it has been reported that p-nitrophenol-glucuronide can serve as the donor of the glucuronyl group by the glucuronidation of o-aminophenol in the presence of UDP and guinea pig liver microsomes[25].

For a heterogeneous group of compounds, glucuronidation is either the only form of metabolic conversion or is a phase II reaction which enables the drug, after some form of oxidation, to be rapidly cleared from the body. Clearance of glucuronides occurs by means of renal excretion and/or secretion into the bile and removal from the body with the feces.

Glucuronides are efficiently cleared by the kidneys. Because of their low lipid solubility, glucuronides are not reabsorbed to a great extent and more specifically, many glucuronides are actively excreted by the kidney tubules[26]. The biliary excretion of glucuronides occurs, presumably, exclusively by means of active processes[27,28].
Why some glucuronides are excreted by the kidneys and others by means of biliary excretion is not yet explained. There is a general tendency for compounds with large molecular weight to be excreted in the bile whereas smaller compounds are excreted by the kidneys, but also other and probably more important factors play a role[29].

The pharmacological activity of glucuronides is low when compared with the parent drug. For example the glucuronides of chloramphenicol, sulfadimethoxine, and 3',5'-dibromosulfanilide have negligible antibacterial activity in vitro[29a], and also the glucuronide of morphine does not have a pharmacological effect in animals[30]. Glucuronides are relatively strong organic acids with pKa values between 3.0 and 4.0[31], as a result of which they are in the ionized state of physioligical pH. This means that they can only penetrate with difficulty to a pharmacological effector site. Furthermore the pharmacological effect of a drug usually depends on a well defined molecular structure[32]. Conjugation with glucu-

ronic acid obviously causes an extensive alteration in this structure, resulting in bioinactivation.

In vivo, the efficiency of glucuronidation as a means of bioinactivation, is strongly influenced by the action of β-glucuronidase. The action of β-glucuronidase in the intestine, for example, causes that some drugs enter an enterohepatic circulation which impairs their disposition. Thus, chloramphenicol glucuronide is deconjugated in the intestine and the released parent compound is further metabolized to toxic arylamines, which are reabsorbed into the circulation[33].
The glucuronidation of the carcinogenic metabolite of 2-naphthylamine, 2-amino-1-naphthol, is an example of an adverse effect of this type of conjugation. The glucuronide of this metabolite is excreted in the urinary tract, where it after deconjugation in the bladder, can cause bladder cancer[34].

UDPglucuronyltransferase, a membrane-bound enzyme

Uridine diphosphate glucuronate glucuronyltransferase (EC 2.4.1.17) is located in the endoplasmic reticulum of the liver cell[10]. It is also present in kidney cortex, gastrointestinal mucosa, epidermis and adrenal tissue. But the activity in these tissues is low compared with the liver[10,35,36,37]. A steroid glucuronyltransferase was found in the outer mitochondrial membrane of pig intestine[38] and in the cytosol fraction of human liver[39].

UDPglucuronyltransferase is bound to the microsomal membranes and there are indications that it is located on the inside of the microsomal vesicles[40,41,42]. After treatment of microsomal preparations with ultrasonication, deoxycholate, digitonin, lubrol or snake venom the enzyme stayed in the supernatant of a 100,000 x g centrifugation[43,44,45,46,47]. Morphological investigation of these so-called solubilized prepara-

tions, showed that they contained membrane fragments[43,44]. Preparations not containing these fragments were devoid of enzyme activity[43]. Thus extraction of UDPglucuronyltransferase from the microsomal membranes apparently results in loss of enzyme activity.
The activity of UDPglucuronyltransferase, in vitro, is greatly influenced by treatment of the microsomes with membrane perturbating agents. Triton X-100[48,49,50], low concentrations of deoxycholate [44,48,51] digitonin[45,52], lubrol[46], tween[53] and ultrasonication[54] enhanced the enzyme activity. Higher activities were also measured after storage of the preparations for several days at $0^{o}C$ or when the homogenization medium contained KCl instead of sucrose[48,55]. In addition, an increase in enzyme activity was observed after a short treatment of microsomal preparations with phospholipase A but this occurred at the expense of the stability of the enzyme[56].

Many functions of UDPglucuronyltransferase seem to depend on the integrity of the enzyme-membrane relationship. In native microsomes for example, UDPglucose, UDPxylose, UDPmannose and UDPgalactose do not interact with the UDPglucuronic acid binding site on the enzyme, but in phospholipase A treated microsomes they inhibit the binding of UDPglucuronic acid[57]. Furthermore stimulation of the enzyme by UDP-N-acetylglucosamine was only found in native microsomes.
UDP-N-acetylglucosamine caused inhibition of the enzyme activity in phospholipase A treated preparations[57]. Also the interaction of sulfhydryl reagents with the enzyme was different in native and phospholipase A treated preparations. Sulfhydryl reagents stimulated the enzyme in a characteristic way in native microsomes, they inhibited the enzyme in phospholipase A treated preparations[58].

The effect of UDP-N-acetylglucosamine on the glucuronyl-

transferase activity in native microsomes is probably mediated by an allosteric mechanism. UDP-N-acetylglucosamine enhances the rate of glucuronidation and increases the affinity for UDPglucuronic acid[59,60]. This effect only occurs in the presence of divalent metal ions[59,60] and could be completely blocked by sulfhydryl reagents[61]. Several glucuronides also may act as allosteric affectors of the enzyme. The glucuronides of 1-naphthol, 2-naphthol and 4-methylumbelliferone stimulate the synthesis of p-nitrophenolglucuronide and increase the affinity for UDPglucuronic acid. p-Nitrophenol glucuronide had a similar effect on the glucuronidation of 1-naphthol, o-naphthol, o-aminobenzoate and bilirubin[62]. These studies were done with native microsomes from guinea pig liver.

Other authors found that in digitonin treated rat liver microsomes, 1-naphtholglucuronide inhibits the glucuronidation of p-nitrophenol and competes with UDPglucuronic acid in this reaction[63]. Thus the allosteric effector site is probably sensitive to membrane perturbating agents. It is also possible that only in detergent treated microsomes glucuronides have free access to the UDPglucuronic acid binding site.

Is lipid required for UDPglucuronyltransferase function ?

Prolonged treatment of microsomes with phospholipase A or C led to inactivation of the p-nitrophenol glucuronidating form of UDPglucuronyltransferase[64]. Products of phospholipase action - fatty acids, lysolecithin, diglyceride and phosphorylcholine - were not inhibitory[64]. The activity of the phospholipase A treated preparation could be completely restored by addition of mixed phospholipid micelles or phosphatidylcholine[65] but the restoration of the phospholipase C treated preparation was only partial[64].

For the steroid glucuronidation the reports are contradictory. Treatment of a solubilized preparation from human liver microsomes with phospholipase C led to inactivation of 16α-oestriol glucuronyltransferase. Lysolecithin, but not e.g. lecithin, partially restorted the activity[68]. Steroid glucuronyltransferase of rabbit liver microsomes was only partially affected by treatment with snake venom and phospholipids only partially reactivated the preparation. Phospholipase C had no effect[67].

The following criteria should be obeyed to determine if an enzyme requires lipid for correct function:

1. correlation between loss of enzymatic activity and alteration or removal of lipid;
2. restoration of activity with addition of lipid; and
3. restoration of activity should be correlated with binding of the lipid to the enzyme[68].

The latter demand can of course only be verified in work with purified enzymes. Microsomal enzymes complying with these criteria are glucose 6-phosphatase, NADH cytochrome b_5 reductase and the microsomal cytochrome P-450 linked hydroxylating system[69,70,1,2,3]. Phosphatidylcholine is specifically required by the reduction of cytochrome P-450[71]. The former two enzymes require phosphatidylethanolamine and phosphatidylcholine, respectively[69,70]. The enzyme mediating the glucuronidation of p-nitrolphenol also complies with the above mentioned criteria. Whether all possible forms of UDP-glucuronyltransferase require lipid for correct function has not been proven yet.

Heterogeneity of UDPglucuronyltransferase

A wide variety of compounds can serve as substrates for UDP-glucuronyltransferase. Steroid hormones, thyroxine and derivatives, catecholamine metabolites, bilirubin and a great

many foreign compounds are conjugated with glucuronic acid before they are suitable acceptor groups for glucuronyl transfer[10,76,77]. The question whether only one aspecific enzyme is responsible for the glucuronidation of these compounds or whether UDPglucuronyltransferase is in fact a group of different enzymes, has been extensivily investigated in recent years.

Steroids are glucuronidated with a high degree of specificity[78]. Studies on the glucuronidation of oestriol have shown that the glucuronyl group was attached to the 3*a*-OH of this oestrogen when guinea pig liver was used as enzyme source[79] but with preparations from mouse or human liver the 16*a*-glucuronide was formed[39,80,81]. In these studies it was also found that the steroid glucuronyltransferase from guinea pig liver does not accept oestriol 16-monoglucuronide as substrate[82]. With mouse liver, however, both unconjugated oestriol as well as oestriol 3-monoglucuronide were substrates for glucuronidation[82]. Furthermore differences in pH optima for different steroid glucuronidations have been reported. Human gut, for example, contains a glucuronyltransferase for the glucuronidation of oestriol, oestradiol and testosterone at the 17β-OH with a pH optimum of pH 6.6[83]. The formation of oestriol 16α-monoglucuronide, oestriol 3-monoglucuronide and oestradiol 3-monoglucuronide however, occurs optimally around pH 8.2[39,78]. These results suggest that different steroid glucuronyltransferase exist. The role of these enzymes, if any, by the glucuronidation of non-steroid compounds is not clear.

The homozygous Gunn rat cannot dispose of its bilirubin because of an autosomal recessive deficiency of the bilirubin glucuronidating enzyme. Studies with this animal revealed that bilirubin glucuronyltransferase is probably a separate entity which differs from the enzymes involved in the synthesis of N-glucuronides, glucuronides of several phenolic

substrates, acyl glucuronides, the glucuronides of thyroxine and derivatives and tetrahydrocortisol glucuronide[85,86,87,88,89,90,91,92]. In contrast to bilirubin glucuronide these latter glucuronides can be formed in the Gunn rat in vivo, or with preparations from the liver of this animal. The disease of the Gunn rat is not confined however to an inability to glucuronidate bilirubin as also relatively low activities were measured by the glucuronidation of some other substrates. The glucuronidation of these substrates, however, could be enhanced to a normal level by pretreatment of the enzyme preparations with diethylnitrosamine[89,90]. An explanation for this phenomenon may be that normally the glucuronidation of these substrates is mediated by the bilirubin glucuronyltransferase, but by deficiency of this enzyme, are glucuronidated by other non-deficient enzymes. These other enzymes apparently do not accept bilirubin as substrate. Accumulation of bilirubin in Gunn rats may cause several additional defects in the liver cell which lead to alterations in the endoplasmic reticulum membrane.
Microsomal membrane preparations from Gunn rats differ from normal rats in that they react differently with detergents[88]. Possibly, related to this, it was also found that the microsomal drug oxidation is disturbed in these animals[93].

The glucuronidation of foreign substrates may be mediated by special enzymes or by forms of UDPglucuronyltransferase for which endogenous compounds are the physiological substrates. From an evolutionary point of view, the latter possibility seems more likely. This problem has been approached by studying inhibition kinetics with alternative substrates or products as inhibitors[94,95,96,97,98]. It has been reported however, that the glucuronidation of several substrates is not according to simple enzyme kinetics. For example, concave Lineweaver Burk plots were found by the glucuronidation of p-nitrophenol and o-aminophenol which may indicate nega-

tive coöperativity by the binding of UDPglucuronic acid[59,99]. Substrate inhibition was found by the glucuronidation of phenolphthalein[98]. Furthermore unexpected interactions may occur by studies on glucuronidation in the presence of alternative substrates. It has for example been reported that the time course for the formation of o-aminophenol glucuronide shows, a distinct upward bend, in the presence of p-nitrophenol[98]. These problems complicate kinetic studies, and the reports are contradictory (compare 94 and 98).

Furthermore the specificity of possible different enzymes may not be absolute for foreign substrates and this also limits the value of these studies. The most convincing evidence with regard to foreign substrates comes from solubilisation studies. Treatment of rabbit liver microsomes with snake venom resulted in a soluble fraction with activity for the formation of p-nitrophenol and anthranilic acid glucuronide, whereas the glucuronidation of aniline remained confined to the insoluble fraction[100], suggesting multiplicity of UDPglucuronyltransferase.

Also the mutual interference by the glucuronidation of foreign substrates and bilirubin has been studied extensively[101,102,103,104,105]. The interpretation of these studies however, is complicated by the following points :

a. Bilirubin is insoluble at pH's generally regarded as optimal for glucuronidation of foreign substrates[106]. Therefore albumin is used to keep bilirubin in solution[101,107,108] but in the presence of albumin kinetic data are hard to evaluate and non-linear Lineweaver Burk plots are found[84,109,110].

b. Bilirubin binds to lipids and membrane[111,112,113] and therefore true substrate concentrations are difficult to calculate.

c. Bilirubin has a detergent-like effect on microsomal membranes, it activates native microsomes and inhibits activa-

ted preparations[114]. Also in these studies contradictory results have been reported (compare 104 and 105), and no conclusive evidence has been obtained about the role of bilirubin glucuronyltransferase in the metabolism of foreign substrates.

Bilirubin glucuronidation

Bilirubin IXα is the physiological breakdown product of heme. Its systemic name is 1'8'-dioxo-1,3,6,7-tetramethyl-2,8,-divinylbiladiene-(a,c)-dipropionic acid-(4,5)[115].
Bilirubin is a lipophilic compound and is insoluble in water below pH 7.8 - pH 8.0[106]. The pKa of the carboxyls is 7.8[116]. Bilirubin readily oxidizes to biliverdin, monopyrroles and dipyrroles[117]. Unconjugated bilirubin is internally stabilized by probably four hydrogen bonds between the carboxyls and the exo pyrrolenone rings in both halfs of the molecule[118,119]. Esterification of the carboxyls of the propionic acid side chains breaks up these bonds and causes that the bilirubin conjugates are extremely labile[119]. Therefore most authors studied the azoderivatives of these conjugates rather than the parent compounds[120,121]. By the analysis of mixtures containing different sugar conjugates this causes the problem of reconstruction of the original tetrapyrrole structures from a mixture of different azodipyrroles. This problem does not exist by in vitro work whereby one deals with only one conjugating group.

In contrast to the traditionally held view that bilirubin is mainly excreted as a diglucuronide[117], it was recently found that a fraction of human T-tube bile[122,123] and bile from dogs[120,121,124], alligators, cats, chickens, horses, opossums, rabbits and snakes[125] consist of alkali-labile, glucuronidase-resistent sugar conjugates, which could be identified as xylosyl and glucosyl conjugates. Furthermore glucuronosyl-glucosyl, glucuronosyl-glucuronosyl and glucosyl-

glucuronosyl conjugates were found and characterized[126]. In all studies both mono- and diconjugates were found[122,123,124,125,126,127]. In vitro, xylosyl and glucosyl conjugates are formed on incubation of bilirubin with UDPxylose or UDP-glucose and preparations from the livers of rats, cats or horses[15,16,125].

Conjugation is an absolute requirement for the disposition of bilirubin. A complete deficiency of the bilirubin conjugating system is a serious inborn error of metabolism (Crigler Najjar, type I). Patients with this disease usually die in the first decade of life, with severe brain damage[128]. None of the above mentioned conjugates is formed in these patients[129].

1. A.Y.H. LU and M.J. COON (1968), J. Biol. Chem., 243, 1331.
2. A.Y.H. LU, K.W. JUNK and M.J. COON (1969), J. Biol. Chem. 244, 3714.
3. W. LEVIN, D. RYAN, S. WEST and A.Y.H. LU (1974), J. Biol. Chem. 249, 1747.
4. T. SCHERSTÉN (1970) in Metabolic conjugation and metabolic hydrolysis, volume 2 (W.H. Fishman ed.) Academic Press, New York.
5. E. BOYLAND and L.F. CHASSEAUD (1969), Advan. Enzymol., 32, 172.
6. M.J. PABST, W.H. HABIG and W.B. JAKOBY (1973), Biochem. Biophys. Res. Comm. 52, 1123.
7. W.H. HABIG, M.J. PABST and W.B. JAKOBY (1974) J. Biol. Chem. 249, in press.
8. K.S. DODGSON and F.S. ROSE (1970) in Metabolic conjugation and metabolic hydrolysis, volume 1 (W.H. Fishman ed.) Academic Press, New York.
9. M. WAKABAYSHI (1970) in Metabolic conjugation and metabolic hydrolysis, volume 2 (W.M. Fishman, ed.) Academic Press, New York.
10. G.J. DUTTON (1966) in Glucuronic acid, free and combined (G.J. Dutton, ed.) Academic Press, New York.
11. R.T. DEAN (1974), Biochem. J. 138, 395.
12. J.L. STROMINGER, E.S. MAXWELL, J. AXELROD and H.M. KALCKAR (1957) 224, 79.
13. A. WHITE, P. HANDLER and E.L. SMITH (1973) Principles of Biochemistry, fifth edition, McGraw-Hill, New York.
14. B. MULLER-OERLINGHAUSEN (1968) in: Ikterus (K.Beck, ed.) F.K. Schattauer Verlag, Stuttgart, New York.

15. J. FEVERY, P. LEROY and K.P.M. HEIRWEGH (1972), Biochem. J. 129, 619.
16. K.P. WONG (1972), Biochem. Pharmacol. 21, 1485.
17. R.S. LABOW, D.G. WILLIAMSON and D.S. LAYNE (1974). Can. J. Biochem. 52, 203.
18. D.G. WILLIAMSON, D.S. LAYNE and D.C. COLLINS (1972) J. Biol. Chem. 247, 3286.
18a. D.A. VESSEY and D. ZAKIM (1973) Biochim. Acta,315, 43.
19. D.C. Collins, H. JIRKU and D.S. LAYNE (1968), J. Biol. Chem. 243, 2928.
20. B. JAKOBSON and E.A. DAVIDSON (1961), Nature, 189, 663.
21. M. HONJO, Y. FURUKAWA, K. IMAI, H. MORIYAMA and K. TANAKA (1962), Chem. Pharm. Bull. (Tokyo) 10, 225.
22. J. AXELROD, J.K. INSCOE and G.M. TOMKINS (1958), J. Biol. Chem. 232, 835.
23. W.H. FISHMAN and S. GREEN (1957), J. Biol. chem. 225, 435.
24. D.A. VESSEY and D. ZAKIM (1972), J. Biol. Chem. 247, 3023.
25. C. BERRY and T. HALLINAN (1974) FEBS letters 42, 73.
26. K.C. HUANG and P.K. KNOEFEL (1957), Federation Proc. 16, 308.
27. L. SPERBER (1959) Pharmacol. Rev. 11, 109.
28. BRAUER (1959) J. Am. Med. Assoc. 169, 1462.
29. P.C. HIROM, P. MILLBURN, R.L. SMITH and R.T. WILLIAMS (1972) xenobiotica 2, 205.
29a. R.L. SMITH and R.T. WILLIAMS (1966) in: Glucuronic acid, free and combined (G.J. Dutton, ed), Academic Press, New York.
30. L.A. WOODS (1954) J. Pharmacol. Exptl. Therap. 112, 158.
31. D. ROBINSON, J.N. SMITH and R.T. WILLIAMS (1953) Biochem. J. 55, 151.
32. A. GOLDSTEIN, L. BRONOW and S.M. KALMAN (1968) in: Principles of Drug Action, Harper and Row, New York.

33. R.W. THOMPSON, M. STURTEVANT, O.D. BIRD and A.J. GLAZKO (1954) Endocrinology, 55, 665.
34. M.J. ALLEN, E. BOYLAND, C.E. DUKES, E.S. HORNING and J.G. WATSON (1957) Brit. J. Cancer 11, 212.
35. G.J. DUTTON and I.H. STEVENSON (1962), Biochim. Biophys Acta 58, 633.
36. A. AITIO (1973) Academic Dissertation, Turku, Finland.
37. I.H. STEVENSON and G.J. DUTTON (1962), Biochem. J. 82, 330.
38. G.S. RAO, R. SCHUMACHER, M.L. RAO and H. BREUER (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 1789.
39. G.S. RAO, M.L. RAO and H. BREUER (1970), Biochem. J. 118, 625.
40. O. HÄNNINEN and R. PUUKKA (1970) Suo. Kemistil. 43, 451.
41. C. BERRY and T. HALLINAN (1974) FEBS letters 42, 73.
42. H. VAINIO (1973) Biochim. Biophys. Acta 307, 152.
43. A.P. MOWAT and I.M. ARIAS (1970) Biochim. Biophys. Acta 212, 65.
44. E. HALAC and A. REFF (1967) Biochim. Biophys. Acta 139, 328.
45. D.H. GREGORY and R.D. STRICKLAND (1973) Biochim. Biophys. 327, 36.
46. R.D. HOWLAND, A. BURKHALTER, A.J. TREVOR, S. HEGEMAN and D.Y. SCHIRACHI (1971). Biochem. J. 125, 991.
47. K.J. ISSELBACHER, M.F. CHRABAS and R.C. QUINN (1962) J. Biol. Chem. 237, 3033.
48. K.K. LUEDERS and E.L. KUFF (1967) Arch. Biochem. Biophys 120, 198.
49. G.J. MULDER (1970) Biochem. J. 117, 319.
50. A. WINSNES (1969) Biochim. Biophys. Acta, 191, 279.
51. B.F.P. ADLARD and G.H. LATHE (1970) Biochem. J. 119, 437.
52. K.P.M. HEIRWEGH and J.A.T.P. MEUWISSEN (1968) Biochem. J. 110, 31p.

53. P.L.M. JANSEN (1972) Neth. J. Int. Med. 15, 207.
54. P.Th. HENDERSON (1971) Life Sci. part II, 9, 511.
55. A.B. GRAHAM and G.C. WOOD (1973) Biochim. Biophys. Acta 311, 54.
56. D.A. VESSEY and D. ZAKIM (1971) J. Biol. Chem. 246, 4649.
57. D. ZAKIM, J. GOLDENBERG and D.A. VESSEY (1973) Eur. J. Biochem. 38, 59.
58. D. ZAKIM and D.A. VESSEY (1972) Arch. Biochem. Biophys 148, 97.
59. D.A. VESSEY, J. GOLDENBERG and D. ZAKIM (1973) Biochim. Biophys. Acta 309, 58.
60. D. ZAKIM, J. GOLDENBERG and D.A. VESSEY (1973) Biochem. Biophys. Res. Commun, 52, 453.
61. A. WINSNES (1971) Biochim. Biophys. Acta 242, 549.
62. D.A. VESSEY and D. ZAKIM (1974) Biochem. J. 139, 243.
63. O. HÄNNINEN and J. MARNIEMI (1971) Eur. Biochem. J. 18, 282.
64. A.B. GRAHAM and G.C. WOOD (1969) Biochem. Res. Comm. 37, 567.
65. D. ATTWOOD, A.B. GRAHAM and G.C. WOOD (1971) Biochem. J. 123, 875.
66. G.S. RAO, M.L. RAO and H. BREUER (1972) J. Steroid Biochem. 3, 1.
67. R.S. LABOW, D.G. WILLIAMSON and D.S. LAYNE (1973) Biochemistry 12, 1548.
68. S. FLEISCHER, G. BRIERLY, H. KLOUQEN and D.B. SLAUTERBACH (1962) J. Biol. Chem. 237, 3264.
69. S.M. DUTTERA, W.L. BYRNE and M.C. GANOZA (1968) J. Biol. Chem. 243, 2216.
70. P.D. JONES and S.J. WAKIL (1967) J. Biol. Chem. 242, 5267.
71. H.W. STROBEL, A.Y.H. LU, J. HEIDEMA and J. COON (1970) J. Biol. Chem. 245, 4851.

72. A. TAUROG, F.N. BRIGGS and I.L. CHAIKOFF (1952) J. Biol. Chem. 194, 655.
73. K.P. WONG and T.L. SOURKES (1968) Biochem. J. 110, 99.
74. G.J. MULDER and A.H. HEGEDOORN (1974) Biochem. Pharmacol. 23, 2101.
75. M.M. AIRAKSINEN, T.A. MIETTINEN and J. HUTTUNEN (1965) Biochem. Pharmacol. 14, 1019.
76. G.J. DUTTON and H.P.A. ILLING, (1972) Biochem. J. 129, 539.
77. H.P.A. ILLING and G.J. DUTTON (1973) Biochem. J. 131, 139.
78. D.S. LAYNE (1970) in Metabolic Conjugation and Metabolic hydrolysis, volume 1 (Fishman, ed.) Academic Press, New York.
79. U. GOEBELSMANN, E. DICZSFALUSY, J. KATZ and M. LEVITZ (1965) Steroids 6, 859.
80. D.A. BOON, N.J. WAGNER and W.R. SLAUNWHITE, unpublished results cit. from R.Y. KIRDANI, W.R. SLAUNWHITE and A.A. SANDBERG (1968) Steroids, 12, 171.
81. W.R. SLAUNWHITE, M.A. LICHTMAN and A.A. SANDBERG (1964) J. Clin. Endocr. 24, 638.
82. R.Y. KIRDANI, W.R. SLAUNWHITE and A.A. SANDBERG (1968) Steroids 12, 171.
83. K. DAHM and H. BREUER (1966) Acta Endocrinol 52, 43.
84. C.H. GUNN (1938) J. Hered. 29, 137.
85. W.D. DRUCKER (1968) Proc.Soc. Exp. Biol. Med. 129, 308.
86. I.M. ARIAS (1961) Biochem. Biophys Res. Comm. 6, 81.
87. H.A.I.M. VAN LEUSDEN, J.A.J.M. BAKKEREN, F. ZILLIKEN and L.A.M. STOLTE (1962) Biochem. Biophys. Res. Comm. 7, 67.
88. D. ZAKIM, J. GOLDENBERG and D.A. VESSEY (1937) Biochim. Biophys. Acta, 297, 497.
89. I. STEVENSON, D. GREENWOOD and J. McEWEN (1968) Biochem. Biophys. Res. Comm. 32, 866.

90. A.P. MOWAT and I.M. ARIAS (1970) Biochem. Biophys. Acta 212, 175.
91. E.V. FLOCK, J.L. BOLLMAN, C.A. OWEN and P.E. ZOLLMAN (1965) Endocrinology 77, 303.
92. N.B. JAVITT (1966), Am. J. Physiol. 211, 424.
93. A.M. GUARINO, R.L. REAGAN and T.E. GRAM (1973) Pharmacology 10, 257.
94. D. ZAKIM, J. GOLDENBERG and D.A. VESSEY (1973) Biochim. Biophys. Acta 309, 67.
95. K.J. ISSELBACHER (1968) in: Ikterus, (K. Beck, ed.) F.K. Schattauer Verlag, Stuttgart.
96. I.D.E. STOREY (1965) Biochem. J. 95, 209.
97. A.R. TEMPLE, M.S. CLEMENT and A.K. DONE (1968) Proc. Soc. Exp. Biol. Med. 128, 307.
98. G.J. MULDER (1971) Biochem. J. 125, 9.
99. A. WINSNES (1972) Brochim. Biophys Acta 284, 394.
100. J. AXELROD, J.K. INSOE and G.M. TOMKINS (1968) J. Biol. Chem. 232, 835.
101. G.H. LATHE and M. WALKER (1968) Biochem. J. 70, 705.
102. G.A. TOMLINSON and S.J. YAFFE (1966) Biochem. J. 99, 507.
103. M. FREZZA, G. DE SANDRE, G. PERONA and R. CORROCHER (1968) Clin. Chim. Acta 21, 509.
104. G.J. MULDER (1972) Biochim. Biophys. Acta 289, 284.
105. D.A. VESSEY, J. GOLDENBERG and D. ZAKIM (1973) Biochim. Biophys Acta 309, 75.
106. R. BRODERSEN and J. THEILGAARD (1969) Scand. J. Clin. Lab. Invest. 24, 395.
107. G.M. GRODSKY and J.V. CARBONE (1957) J. Biol. Chem. 226, 449.
108. F.P. VAN ROY and K.P.M. HEIRWEGH (1968) Biochem. J. 107, 507.
109. K.P.M. HEIRWEGH, M. VAN DE VIJVER and J. FEVERY (1972) Biochem. J. 129, 6060.

110. M. BLACK, B.H. BILLING and K.P.M. HEIRWEGH (1970) Clin. Chim. Acta 29, 27.
111. M.G. MUSTAFA and T.E. KING (1970) J. Biol. Chim. 245, 1084.
112. G.B. ODELL (1965) Proc. Soc. Exp. Biol. Med. 120, 352.
113. D. BRATLID (1972) Scand. J. Clin. Lab. Invest. 29, 91.
114. E. SANCHEZ and T.R. TEPHLY (1973) Life Sci. 13, 1483.
115. W. RÜDIGER (1972) in: Progress in the chemistry of organic natural compounds, volume 29 (W. Herz, H. Grisebach and G.W. Kirby, eds.). Springer Verlag, Wien, New York.
116. J. KRASNER and S.J. YAFFE (1973) Biochem. Med. 7, 128.
117. T.K. WITH (1960), in Biologie der gallenfarbstoffe, Thieme Verlag Stuttgart.
118. C.C. KUENZLE, R.R. PELLONI and M.H. WEIBEL (1972) Biochem. J. 130, 1147.
119. C.C. KUENZLE, M.H. WEIBEL and R.R. PELLONI (1973) Biochem. J. 133, 357.
120. F. COMPERNOLLE, G.P. VAN HEES, J. FEVERY and K.P.M. HEIRWEGH (1971) Biochem. J. 125, 811.
121. J. FEVERY, G.P. VAN HEES, P. LERY, F. COMPERNOLLE and K.P.M. HEIRWEGH (1971), Biochem. J. 125, 803.
122. K.P.M. HEIRWEGH, G.P. VAN HEES, P. LERY, F.P. VAN ROY and F.H. JANSEN (1970) Biochem. J. 120, 877.
123. J. FEVERY, B. VAN DAMME, R. MICHIELS, J. DE GROOTE and K.P.M. HEIRWEGH J. Clin. Invest 51, 2482.
124. E.R. GORDON, M. DADOUN, C.A. GORESKY, T.H. CHAN and A.S. PERLIN (1974) in Press.
125. C.E. CORNELIUS, K.C. KELLEY and J.A. HIMES (1974). J. Vet., in press.
126. C.C. KUENZLE (1970) Biochem. J. 119, 387; Biochem. J. 119, 395; Biochem. J. 119, 411.
127. F.H. JANSEN and B.H. BILLING (1971) Biochem. J. 125, 917.

128. I.A. ARIAS, L.M. GARTNER, M. COHEN, J. BEN EZZER and A.J. LEVI (1969) Am. J. Med. 47, 395.

129. I.A. ARIAS, personal communication.

130. P. ZEIDENBERG, S. ORRENIUS and L. ERNSTER (1967) J. Cell Biol. 32, 528.

PLAN AND INTENT OF STUDY

The glucuronyl conjugation is the subject of this study. In particular the question whether UDPglucuronyltransferase is one enzyme, catalyzing the glucuronidation of many different compounds, or a multiplicity of enzymes, catalyzing more or less specific reactions, was investigated.

In paper I, the glucuronidation of different substrates was studied before and after treatment of the test-animals with phenobarbital, for different periods of time. The assumption was made that if the glucuronidation of p-nitrophenol and bilirubin is mediated by different enzymes, their response to the inducing effect phenolbarbital may be different. Although it is known that the effect of treatment with phenobarbital on the metabolism in the liver cell is specific in that it increase the total amount of drug metabolizing smooth endoplasmic reticulum, on the other hand induction of some enzymes is more rapidly achieved than of others[130]. This probably depends on differences in turnover rates of the enzyme proteins and on differences at the regulatory level. Animals were choosen which are known extremes for the glucuronidation of the studied compounds.

The papers II, III and IV deal with the bilirubin glucuronidating form of UDPglucuronyltransferase. Bilirubin and bilirubin monoglucuronide are both physiological substrates for glucuronidation, and may be specific substrates for certain forms of UDPglucuronyltransferase. In view of the great specificity found by the glucuronidation of steroids, it seemed worthwhile to study these two endogenous substrates. The glucuronidation of bilirubin monoglucuronide, in vitro, has not been studied before.

Paper V reports on the solubilization, delipidation and re-

constitution of UDPglucuronyltransferase. The hypothesis of this study was that solubilization of the enzyme-protein from the microsomal membranes may result in an inactive preparation. Reconstitution of the enzyme with detergents or phospholipids was thought to be an essential step by its purification.

Publications :

I. *P.L.M. Jansen and P.Th. Henderson* : Influence of phenobarbital treatment on p-nitrophenol and bilirubin glucuronidation in wistar rat, Gunn rat and cat. Biochem. Pharmacol. (1972) *21*, 2457-2462.

II. *P.L.M. Jansen* : Mono- and diglucuronidation of bilirubin. Folia. Med. Neerl. (1972) *15*,205-213. Presented at the meeting of the European Association for the study of the liver, Arnhem, september 1972.

III. *P.L.M. Jansen* : The isomerisation of bilirubin monoglucuronide. Clin. Chim. Acta, (1973) *49*, 233-240.

IV. *P.L.M. Jansen* : The enzyme-catalized formation of bilirubin diglucuronide by a solubilized preparation from cat liver microsomes. Biochim. Biophys. Acta, (1974) *338*, 170-182.

V. *P.L.M. Jansen and I.M. Arias* : Delipidation and reactivation of UDPglucuronyltransferase from rat liver. Submitted for publication (1974).

INFLUENCE OF PHENOBARBITAL TREATMENT ON *p*-NITROPHENOL AND BILIRUBIN GLUCURONIDATION IN WISTAR RAT, GUNN RAT AND CAT

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Abstract—The influence of phenobarbital pretreatment on the glucuronidation of *p*-nitrophenol and bilirubin has been compared in liver microsomes from Wistar rat, homozygous and heterozygous Gunn rat and cat Both for Wistar rat and cat it appeared that phenobarbital has a different effect on *p*-nitrophenol and bilirubin glucuronidation No bilirubin glucuronidating activity could be detected in homozygous Gunn rats both before and after exposure to phenobarbital The *p*-nitrophenol glucuronidating capacity of Gunn rats, however, was strongly enhanced after phenobarbital treatment. These results are arguments in favour of the involvement of different enzymes in the glucuronidation of *p*-nitrophenol and bilirubin

UDPGLUCURONYLTRANSFERASE (UPDglucuronate glucuronyltransferase, EC 2 4 1 17, acceptor unspecific) catalyzes the transfer of the glucuronyl group from glucuronic acid to aglycones. Ether-, ester-, N- and S-glucuronides are thus formed.

There are many indications that UDPglucuronyltransferase consists of a heterogeneous group of enzymes Homozygous Gunn rats, for instance, although deficient in bilirubin glucuronidation, have a normal glucuronidating capacity towards *p*-nitrophenol, aniline and tetrahydrocortisone. The cat, however, has been reported to have a normal bilirubin metabolism accompanied by a poor capacity to glucuronidate *p*-nitrophenol, menthol and *o*-aminophenol (summarized by Dutton[1]) In this study Wistar rats, homozygous and heterozygous Gunn rats and cats were treated with phenobarbital The effect of this treatment on the *in vitro* activity of UDPglucuronyltransferase, with *p*-nitrophenol and bilirubin as substrates, has been studied

It is known that treatment with phenobarbital stimulates liver growth and increases the amount of microsomal protein in the liver [2] In order to differentiate between these effects and those on the *p*-nitrophenol and bilirubin glucuronidation, an increase of specific activity was considered to be a prerequisite for enzyme induction

Mulder[3] and Winsnes[4] have reported that phenobarbital does not stimulate *p*-nitrophenol glucuronidation unless activated enzyme preparations are used In this investigation microsomes were activated with ultrasonic vibration which has a similar effect as Triton X-100, used by the latter authors [5]

METHODS

Animals. Male Wistar and Gunn rats, 3–4 months old, weighing about 250 g, and male cats, approx 1 year old, weighing about 2500 g were used.

Gunn rats were obtained from the University of California in 1958 and bred in our

Animal Laboratory. Icteric homozygous males were mated with nonicteric heterozygous females.

Phenobarbital treatment. Wistar and Gunn rats were treated daily with 75 mg and cats with 40 mg phenobarbital/kg body wt. for 6 days.

Wistar rats and cats treated for 12 days successively received daily 20 mg/kg for 3 days, 30 mg/kg for 3 days and 40 mg/kg for 6 days. The Gunn rats received daily, 75 mg phenobarbital/kg body wt. for 12 days. Control animals received saline. The animals were injected intraperitoneally, once daily at 9 a.m. Enzyme activities were determined 24 hr after the last injection.

Preparation of microsomes. The rats were killed by stunning and decapitation, the cats by injecting 4 ml sodium pentobarbital (6 per cent) into the heart, immediately followed by decapitation. After the animals were exsanguinated the livers were excised, weighed and kept in ice.

Portions of the livers were weighed, finely minced and transferred into 4 vol. of an ice-cold 0·25 M sucrose solution, containing 10^{-3} M sodium ethylenediamine tetra-acetate (EDTA–Na) and 5×10^{-2} M tris(hydroxymethyl)aminomethane–HCl buffer (pH 7·4).

Homogenates of rat liver were prepared using a Teflon-glass Potter–Elvehjem homogenizer. Cat liver was homogenized with an Ultra-Turrax apparatus.

Microsomal fractions were obtained by centrifuging the homogenates at 9000 *g* in an IEC B-20 centrifuge at 2° for 20 min. The 9000 *g* supernatants were centrifuged at 105,000 *g* in a Christ Omega II ultracentrifuge at 2° for 1 hr. The pellets from the latter step were gently resuspended in the homogenization medium with the Potter–Elvehjem apparatus. One ml suspension contained microsomes derived from 1 g liver.

The suspensions were treated with an ultrasonic distintegrator (MSE-100 Watt) for 6×10 sec, at maximal output.

Chemicals. Bilirubin, ascorbic acid, sulfanilic acid and sodium nitrate were obtained from Merck A. G., Germany; *p*-nitrophenol from the British Drug Houses Ltd., England; the disodium salt of UDPglucuronate from C. F. Boehringer, Germany. Bovine serum albumin was obtained from Poviet, The Netherlands.

Enzyme assay. Bilirubin was dissolved in calcium-free Tyrode buffer (pH 9·0). The incubation system consisted of: 0·30 ml microsomal preparation, 0·80 ml Tris–HCl buffer (0·05 M, pH 7·4), 0·15 ml UDP glucuronate (6×10^{-3} M, final conc.), 0·05 ml $MgCl_2$ ($3{\cdot}3 \times 10^{-3}$ M, final conc.) and 0·20 ml bilirubin ($20–80 \times 10^{-6}$ M, final conc.). The products were assayed according to the method described by Lathe and Walker[6] using diazotized sulphanilic acid as the diazo reagent. Blanks containing all above mentioned ingredients, except UDPglucuronate, were submitted to exactly the same diazo procedure as the reagent mixtures.

The *p*-nitrophenol incubation system consisted of: 0·05 ml microsomal preparation, 0·10 ml $MgCl_2$ ($0{\cdot}5 \times 10^{-3}$ M, final conc.), 0·05 ml UDPglucuronate (6×10^{-3} M, final conc.) and 0·05 ml *p*-nitrophenol ($0{\cdot}5–6 \times 10^{-3}$ M, final conc.) and the reagents were dissolved in Tris–HCl buffer (0·05 M, pH 7·4). The assay was done as described by Henderson and Kersten.[7] The reaction mixtures were incubated at 37° for 30 min.

Protein determination. Protein was determined by the method of Lowry *et al.*[8] with bovine serum albumin as reference.

Calculations. Kinetic data were analysed using Lineweaver–Burk plots. Michaelis–

Menten constants (K_m) are called apparent because crude microsomal preparations were used. Maximal substrate turnover rates per mg microsomal protein (t_{max}) were calculated by extrapolation. Statistical analysis was done with Student's t-test.

RESULTS

The results are summarized in Fig. 1 and Table 1. We ascertained that rates of conversion for both p-nitrophenol and bilirubin remained unchanged during the incubation period.

Wistar rats. p-Nitrophenol glucuronidation appeared to be induced after a 6 day treatment with high doses phenobarbital whereas no induction of the bilirubin glucuronidation was found. Both p-nitrophenol and bilirubin glucuronidation were induced after a 12 day treatment with low doses phenobarbital.

Concomitant with increased t_{max}-values apparent K_m-values were also increased (Fig. 1).

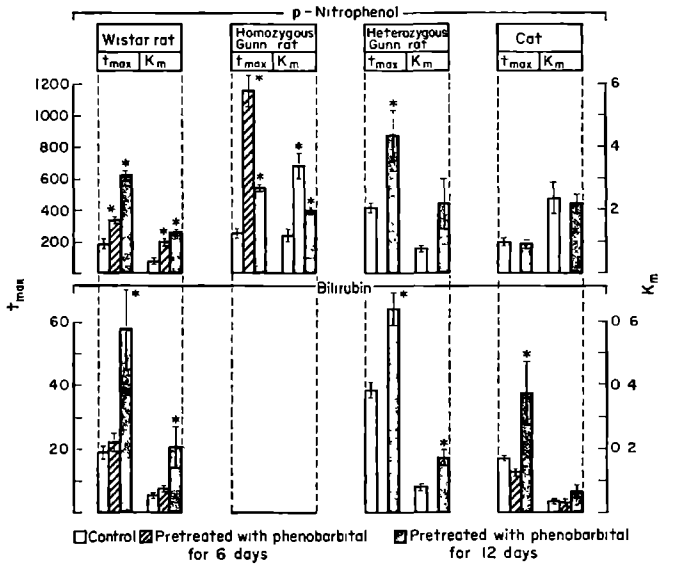

FIG. 1. Influence of phenobarbital treatment upon apparent K_m and t_{max} of p-nitrophenol and bilirubin glucuronidation in Wistar rat, homozygous and heterozygous Gunn rat and cat.

The number of animals and dosage are described in Table 1. Blocks represent means ± S.E.

* $P < 0{\cdot}05$ (Student's t-test). t_{max} and K_m are expressed as nmoles/hr per mg protein and mM respectively.

Increments of microsomal protein were found after phenobarbital treatment but liver wt./body wt. ratio was not changed (Table 1).

Homozygous Gunn rats. A total deficiency in the glucuronidation of bilirubin was found together with high activities for the glucuronidation of p-nitrophenol. After 6 and 12 days phenobarbital treatment the t_{max} for the glucuronidation of p-nitro-

TABLE 1. INFLUENCE OF PHENOBARBITAL TREATMENT ON MICROSOMAL PROTEIN AND LIVER WT /BODY WT. RATIO IN WISTAR RAT, GUNN RAT AND CAT

	Days treated with phenobarbital*	*n*	Microsomal protein† (mg/g wet liver wt)	$\frac{\text{Liver wt}}{\text{Body wt}} \times 100$†
Wistar rat	Control	6	22 9 ± 0 7	4 3 ± 0 5
	6	6	26 4 ± 0 9§	5 0 ± 0 5
	12	6	25 8 ± 0 4§	5 1 ± 0 5
Homozygous Gunn rat	Control	6	18 1 ± 1 3	4 0 ± 0 7
	6	6	25 5 ± 2 1§	5 4 ± 0 9
	12	6	26 6 ± 1 8§	4 5 ± 0 5
Heterozygous Gunn rat	Control	7	18 8 ± 1 0	3 7 ± 0 3
	12	4	19 8 ± 1 7	3 9 ± 0 4
Cat	Control	6	12 5 ± 1 3	3 0 ± 0 3
	6	3	15 3 ± 2 7	3 3 ± 1 2
	12	3	13 4 ± 0 8	4 2 ± 0 3‡

* Wistar rats and homozygous Gunn rats were treated daily with 75 mg phenobarbital/kg body wt for 6 days; cats with 40 mg/kg for 6 days Wistar rats and cats treated for 12 days received daily, successively, 20 mg/kg for 3 days, 30 mg/kg for 3 days and 40 mg/kg for 6 days The Gunn rats received daily 75 mg/kg for 12 days Control animals received saline

n, number of animals in each group.

† means ± S.E

‡ 0 01 < P < 0 05

§ P < 0 01.

phenol amounted respectively to about 450 and 200 per cent of the control value. The same pattern was observed for the apparent K_m-value (Fig. 1).

The influence of phenobarbital treatment upon microsomal protein and liver wt./body wt. ratio was the same as found for the Wistar rat (Table 1).

Heterozygous Gunn rats. The t_{max}-values of both *p*-nitrophenol and bilirubin glucuronidation were enhanced after 12 days treatment with phenobarbital but only the apparent K_m for the bilirubin glucuronidation was concomitantly increased (Fig. 1). Neither microsomal protein nor liver wt /body wt ratio were increased after phenobarbital treatment (Table 1).

Cats *p*-Nitrophenol glucuronidation could not be induced by phenobarbital treatment. The t_{max}-value of the bilirubin glucuronidation was increased after a 12 day phenobarbital treatment whereas no induction was found after 6 days. In both cases phenobarbital treatment did not result in increased apparent K_m-values

Phenobarbital treatment had no influence on the microsomal protein of cat liver. Liver wt./body wt. ratio was increased only after 12 days (Table 1).

DISCUSSION

The question whether or not UDPglucuronyltransferase is one enzyme has never definitely been resolved Mulder[9] and Storey[10] studying the competition between several aglycones, like *p*-nitrophenol, *o*-aminophenol, phenolphthalein and anthranilic acid, came to contradictory conclusions. Assuming the existence of several glucuronyltransferases however, these enzymes might be quite unspecific towards these

substrates. Tomlinson and Yaffe[11] and Halac and Reff[12] tried to solubilize glucuronyltransferase from crude liver microsomes with detergents and reported that different results were obtained with *p*-nitrophenol and bilirubin as substrates. Moreover a different post-natal development[11] and a different behaviour towards heat denaturation[12] was found with these two substrates.

In the present study it is shown that the hereditary lack of the bilirubin glucuronidation enzyme of the homozygous Gunn rat is total. This is at variance with the data of other authors[14] who reported low activities in these animals In contrast the *p*-nitrophenol glucuronidating activity was not deficient. This is in agreement with the results of Van Leusden *et al*,[13] who used Gunn rats of the same breeding colony. The *p*-nitrophenol-glucuronidating capacity of the homozygous Gunn rats increased strongly after phenobarbital treatment. It appeared that the *p*-nitrophenol-glucuronidating enzyme does not accept bilirubin as a substrate since no bilirubin glucuronidation was found after 6 days of phenobarbital treatment whereas the amount of *p*-nitrophenol-glucuronidating enzyme was abundant. This pleads for the existence of a separate bilirubin-glucuronidating enzyme-system which is lacking in the homozygous Gunn rat. This bilirubin-glucuronidating enzyme must also be different from the enzyme(s) glucuronidating aniline, tetrahydrocortisone, *o*-aminophenol, phenoldibromphthalein and diphenylacetic acid, in which the homozygous Gunn rat is not deficient.[15–18]

p-Nitrophenol and bilirubin glucuronidation were influenced differently by phenobarbital, both in Wistar rat and cat. In the Wistar rat *p*-nitrophenol glucuronidation was induced after a 6 day treatment with high doses, whereas bilirubin glucuronidation was only found to be induced after a 12 day treatment. This was achieved with relatively low doses phenobarbital.

Our finding that *p*-nitrophenol glucuronidation is elevated in rats after a short treatment with high doses phenobarbital is in agreement with the results of Mulder [3] As far as bilirubin glucuronidation is concerned, Pilcher *et al* [19] reported that induction was only found after treating rats for a long period with high doses phenobarbital whereas no increase was found after treatment for a short period. These authors however found a decreased specific activity for the bilirubin glucuronidation after 30 mg phenobarbital/kg for 10 days As they used for the *in vitro* assay albumin as a carrier for bilirubin the effective substrate concentration might have been much lower than the calculated one, due to the strong binding of bilirubin to albumin.[20] A rise in apparent K_m after phenobarbital treatment might then explain their results.

In the cat phenobarbital treatment did not effect *p*-nitrophenol glucuronidation nor microsomal protein whereas bilirubin glucuronidation was induced Compared with the Wistar rat, *p*-nitrophenol glucuronidation by the cat appears to be less efficient: beside an equal t_{max} a remarkably higher apparent K_m was found for the glucuronidation of this compound in the cat This might explain the absence of glucuronides in the urine of cats after injection of glucuronidogenic compounds [21] Presumably in the cat glucuronidation is not a preferential pathway for the elimination of foreign compounds

These distinct effects of phenobarbital on the glucuronidation of *p*-nitrophenol and bilirubin in both Wistar rat and cat add to the evidence that *p*-nitrophenol and bilirubin are glucuronidated by different enzymes. Compared with the influence of phenobarbital treatment on the microsomal oxydative enzymes,[2] *p*-nitrophenol

glucuronidation and microsomal protein,[2] the induction of the bilirubin-glucuronidating enzyme is a slow effect The question arises whether this is still a direct effect of phenobarbital treatment or rather some secondary effect It is known that bilirubin production is increased after phenobarbital treatment [22] Prolonged exposure to a higher bilirubin level might induce the bilirubin-glucuronidating enzyme, as was found in newborn rats [23]

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REFERENCES

1 G J Dutton, in, *Glucuronic acid, free and combined* (Ed G J Dutton), p 230 Academic Press, New York (1966)
2 A H Conney, *Pharmac Rev* **19,** 317 (1967)
3 G J Mulder, *Biochem J* **117,** 319 (1970)
4 A Winsnes, *Biochem Pharmac* **20,** 1853 (1971)
5 P Th Henderson, *Life Sci* **9,** 511 (1970)
6 G H Lathe and M Walker, *Biochem J* **70,** 705 (1958)
7 P Th Henderson and K J Kersten, *Biochem Pharmac* **19,** 2343 (1970)
8 O H Lowry, N J Rosebrough A L Farr and R J Randall, *J biol Chem* **193,** 265 (1951)
9 G J Mulder, *Biochem J* **125,** 9 (1971)
10 I D E Storey, *Biochem J* **95,** 209 (1965)
11 G A Tomlinson and S J Yaffe, *Biochem J* **99,** 507 (1966)
12 E Halac and A Reff, *Biochem biophys Acta* **139,** 328 (1967)
13 H A I M van Leusden, J A J M Bakkeren, F Zilliken and L A M Stolte, *Biochem biophys Res Commun* **7,** 67 (1962)
14 A E White, in, *Bilirubin metabolism* (Ed I A D Bouchier), p 183 Blackwell Scientific, Oxford (1967)
15 I M Arias, *Biochem biophys Res Commun* **6,** 81 (1961)
16 W D Drucker, *Proc Soc exp Biol Med* **129,** 308 (1968)
17 I Stevenson, D Greenwood and J McEwen, *Biochem biophys Res Commun* **32,** 866 (1968)
18 N B Javitt, *Am J Physiol* **211,** 424 (1966)
19 C W T Pilcher, R P H Thompson and R Williams *Biochem Pharmac* **21,** 129 (1972)
20 D Bratlid and J Fog, *Scand J clin Lab Invest* **25,** 257 (1970)
21 D Robinson and R T Williams *Biochem J* **68,** 23P (1958)
22 R J Roberts and G J Plaa, *Toxicol Appl Pharmac* **15,** 483 (1969)
23 A F Bakken, *Pediat Res* **3,** 205 (1969)

Mono- and diglucuronidation of bilirubin

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UDPglucuronyl transferase in the endoplasmic reticulum of the liver cell catalyses the transfer of the glucuronyl group from UDPglucuronic acid to physiological compounds such as bilirubin, thyroxine, adrenaline and steroid hormones, and also to many compounds foreign to the organism, *e g* drugs There are many indications that UDPglucuronyl transferase consists of a heterogeneous group of enzymes[4]

The homozygous Gunn rat is jaundiced because it is unable to glucuronidate bilirubin, on the other hand, many other compounds are reported to be glucuronidated by this animal[4]. Bilirubin glucuronyl transferase must therefore be different from the glucuronidating enzymes for which the Gunn rat is not deficient, and which presumably do not accept bilirubin as a substrate Bilirubin was therefore chosen as substrate in our study on glucuronidation processes in the liver.

Since in bile bilirubin appears mainly conjugated with two glucuronic acid molecules[5,7], we examined how this diglucuronide is formed enzymatically It was found that, *in vitro*, the two glucuronyl groups are attached to the bilirubin molecule in two steps first, bilirubin monoglucuronide is formed and subsequently bilirubin monoglucuronide is conjugated to bilirubin diglucuronide Whether these two steps are mediated by one or two enzymes was studied by investigating the glucuronidation of bilirubin or bilirubin monoglucuronide in the presence of *p*-nitrophenol, which is a good substrate for glucuronidation

METHODS

Chemicals – Triton X-100 was obtained from BDH Chemicals Ltd , Poole, England, polyoxyethylene sorbitan monooleate (Tween 80) from Applied Science Laboratories Inc , State College, Pa , USA, deoxycholic acid sodium salt from Sigma, St Louis, Mo , USA, and Sephadex G-25 (coarse) from Pharmacia, Uppsala, Sweden Bilirubin was purchased from Merck A G. Germany, UDPglucuronate (disodium salt) from Boehringer, Germany, *p*-nitrophenol from BDH Chemicals Ltd , England, and saccharo-1 4-lactone from Calbiochem , Los Angeles, USA *o*-Ethylanthranilate came from Eastman Organic Chemicals, Rochester, N Y , USA, and bovine serum albumin from Poviet, Amsterdam, The Netherlands In thin-layer chromatography, precoated silica gel plates, layer thickness 0 25 mm, from Merck A G , Germany, were used

Animals – Male cats, about 1 year old, and male Wistar rats, about 3 months old, were used

Preparation of microsomes – 20% (wt/vol) Homogenates of cat and Wistar rat livers were prepared in 0 25 M sucrose, 10^{-3} M EDTA-Na, 0 05 M Tris-HCl buffer (pH 7 8) After centrifugation at 9000 g for 20 min the supernatant fraction was centrifuged at 105,000 g for 1 hr Pellets were resuspended in the homogenization medium so that 1 ml suspension contained microsomes derived from 1 g liver Triton X-100 (0 03%-1 7%, vol/vol) or Tween 80 (0 5%-7 5% vol/vol) was added to these microsomal suspensions

Solubilization of UDPglucuronyl transferase from cat liver microsomes – Microsomes were resuspended in 0 3% deoxycholate dissolved in the abovementioned homogenization medium After stirring for 4 hrs at 4°C the suspension was centrifuged at 105,000 g for 1 hr The supernatant was passed over a column (16 × 1000 mm) containing Sephadex G-25

Enzyme assays

a Conversion of bilirubin to bilirubin mono- and diglucuronide ($B \rightarrow \overline{BG + BG_2}$) – Reagent mixtures contained 200 µl bilirubin (aqueous solution), 200 µl UDPglucuronate, 200 µl enzyme and 600 µl Tris-HCl buffer (0 05 M, pH 8 3) containing $MgCl_2$ (3 3 mM) and saccharo-1 4-

lactone (1 mM) Final concentrations are mentioned in the legends of figures and Tables The assay was done as described by BLACK *et al*[2] The colour of the azopigment mixture, obtained after reaction of glucuronidated bilirubin with the diazonium salt of *o*-ethylanthranilate, was determined spectrophotometrically at 530 nm Conjugated bilirubin was calculated, assuming ϵ_{530} 44 4 × 10^3 $M^{-1}cm^{-1}$ (according to VAN ROY and HEIRWEGH[12])

b Conversion of bilirubin to diglucuronide (B → BG → $\overline{|BG_2|}$) – The same assay and incubation system was used as mentioned under a The amount of diglucuronide was determined by applying a sample of the azopigment mixture directly to the silica gel plates Chromatograms were developed with chloroform methanol water (65 25 3, vol/vol/vol) Two clearly defined spots appeared in the α_0 and δ region, representing azobilirubin and azobilirubin monoglucuronide, respectively[5] Diazotized rat bile was used as a reference. The spots were eluted in 1 ml methanol and the extinction was read at 530 nm in a Zeiss spectrophotometer PMQ II Freshly prepared blanks containing the reagents mentioned under a, except UDPglucuronate, were submitted to exactly the same diazo reaction as the reagent mixtures. Their chromatograms showed only a very faint diazo-positive line in the α_0 region indicating that unconjugated bilirubin does not react with diazotized *o*-ethylanthranilate The following calculations were made % monoglucuronide = % α_0 + % equimolar δ − 2 × % α_0, % diglucuronide − % residual δ = % δ − % α_0 (adapted from ref 11)

c. Conversion of bilirubin monoglucuronide to diglucuronide (BG → $\overline{|BG_2|}$) – In a previous study it was found that after incubation of Wistar rat liver microsomes with bilirubin and UDPglucuronate (in low concentration), bilirubin monoglucuronide was formed in great excess[7] Therefore an incubation system containing Wistar rat microsomes was used to generate bilirubin monoglucuronide This bilirubin monoglucuronide was then used as a substrate for incubation with solubilized enzyme derived from cat liver microsomes Incubation media used for the generation of bilirubin monoglucuronide contained 2 ml bilirubin (166 μM) 2 ml UDPglucuronate (2 mM), 2 ml with Triton X-100 (0 25%, vol/vol) pre-treated microsomes derived from Wistar rat liver, and Tris-HCl buffer (0 05 M, pH 8 3) containing $MgCl_2$ (3 3 mM) After incubation for 30 min at 37°C, bilirubin was extracted with chloroform The chloroform phase was discarded after centrifugation and the protein layer, which appeared to contain most of the bilirubin monoglucuronide, was suspended in the aqueous phase The amount of bilirubin monoglucuronide in this suspension was determined as described under b

0 8 ml Monoglucuronide-containing suspension was incubated with 0 2 ml UDPglucuronate (3 mM) and 0 2 ml solubilized enzyme preparation derived from cat liver for 25 min at 37°C Bilirubin monoglucuronide and bilirubin diglucuronide were determined as described under b

d p-Nitrophenol glucuronidation – The assay was according to HENDERSON and KERSTEN[8] Incubations were done with the solubilized enzyme preparation at pH 8 3, in media containing $MgCl_2$ (3 3 mM)

Protein determination – Protein was determined by the method of LOWRY *et al*[9], with bovine serum albumin as referenc

RESULTS

Both bilirubin mono- and diglucuronide were formed when bilirubin and UDPglucuronate were incubated with microsomes derived from cat liver Besides Triton X-100, Tween 80 also appeared to activate microsomal bilirubin glucuronidation (Fig 1) With respect to the amount of bilirubin glucuronidated (thus the amount of monoglucuronide + diglucuronide formed) Triton and Tween were equally potent activating agents A greater fraction of glucuronidated bilirubin, however, was in the diglucuronide form when Tween was present in the incubation medium

The effect of albumin on the glucuronidation of bilirubin is shown in Fig 2 Compared with the total amount of glucuronidated bilirubin, relatively less diglucuronide was formed when albumin was present in low concentrations (9 mg/ml)

To study the enzyme kinetics of the formation of bilirubin mono- and diglucuronide, the glucuronidating enzyme system was solubilized from cat liver microsomes (Table I) Glucuronidation

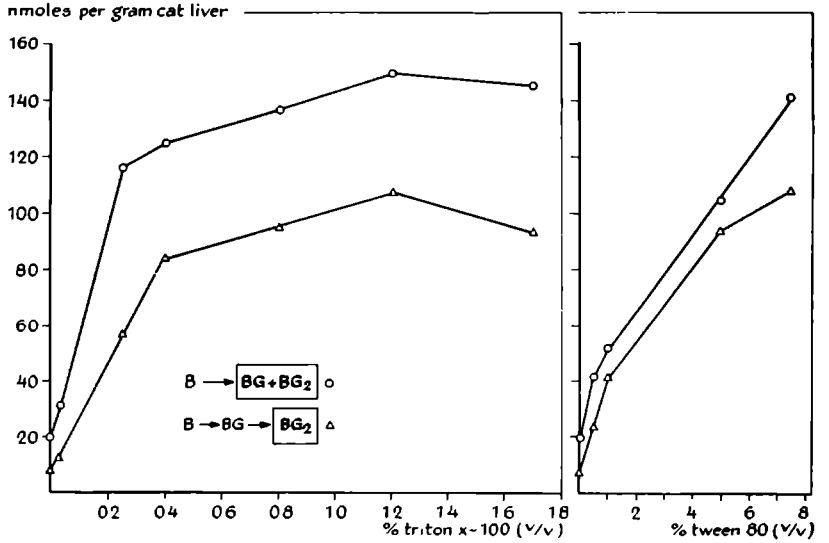

Fig. 1. Influence of Triton X-100 and Tween 80 on the conversion of bilirubin to bilirubin mono- + diglucuronide ($B \rightarrow \boxed{BG + BG_2}$) and on the conversion of bilirubin to bilirubin diglucuronide ($B \rightarrow BG \rightarrow \boxed{BG_2}$). Reagent mixtures consisted of: 200 µl bilirubin (80 µM), 200 µl UDPglucuronate (5 mM), 200 µl microsomal suspension and 600 µl Tris-HCl buffer (0.05 M, pH 8.3) containing $MgCl_2$ (3.3 mM) and saccharo-1.4-lactone (1 mM). Detergents were added to the microsomal suspension 15 min prior to incubation. Detergent concentrations (abscissa) refer to the concentration in the microsomal suspension. The ordinate represents nmoles product formed per g cat liver after 30 min. Determinations are described under a. and b. (Methods).

TABLE I: SOLUBILIZATION OF BILIRUBIN GLUCURONYL TRANSFERASE FROM CAT LIVER

	Protein (mg/ml)	*$B \rightarrow \boxed{BG + BG_2}$* specific activity (nmoles/hr mg protein)*	*$BG \rightarrow \boxed{BG_2}$** specific activity (nmoles/hr mg protein)*
9000 g supernatant	24.5	1.0	1.1
105,000 g supernatant	19.5	–	–
105,000 g pellet	15.9	4.0	2.5
Deoxycholate supernatant	14.0	9.2	5.0

* $B \rightarrow \boxed{BG + BG_2}$: Conversion of bilirubin to mono- + diglucuronide was determined as described under a. (see Methods). Reagent mixtures consisted of: 200 µl bilirubin (166 µM), 200 µl UDPglucuronate (5 mM), 200 µl enzyme preparation and 600 µl Tris-HCl buffer (0.05 M, pH 8.3) containing $MgCl_2$ (3.3 mM) and saccharo-1.4-lactone (1 mM). Incubation for 30 min at 37°C.

** $BG \rightarrow \boxed{BG_2}$: Conversion of bilirubin monoglucuronide to diglucuronide. Substrate was generated as described under c., and determined as described under b. (Methods). Reagent mixtures contained: 800 µl bilirubin monoglucuronide solution (25 µM), 200 µl UDPglucuronate (3 mM) and 200 µl enzyme preparation. Incubation for 25 min at 37°C. The amount of bilirubin diglucuronide was determined as described under b. (Methods).

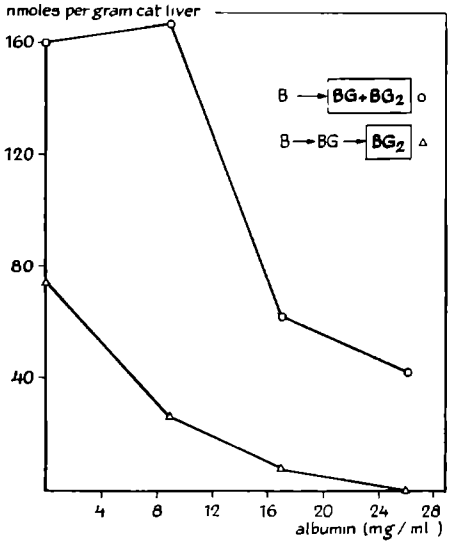

Fig. 2 Influence of albumin on the conversion of bilirubin to bilirubin mono- and diglucuronide (B → BG + BG2) and on the conversion of bilirubin to bilirubin diglucuronide (B → BG → BG2) Reagent mixtures are described in the legend of Fig 1 Bilirubin concentration was in this experiment 166 μM Microsomes were pre-treated with Triton X-100 (1 2%, vol/vol) Abscissa albumin in final concentration, ordinate nmoles product formed per g cat liver after 30 min Determinations are described under a and b (Methods)

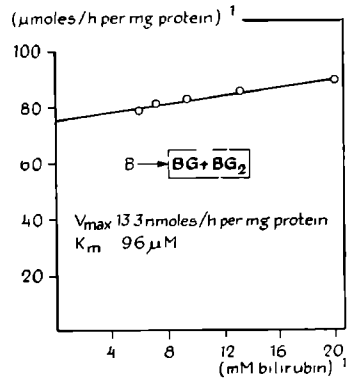

Fig 3 Glucuronidation of bilirubin (B → BG + BG2) 1/reaction velocity is plotted against 1/substrate concentration according to LINEWEAVER and BURK[3] Reagent mixture is described in the legend of Fig 1 A solubilized enzyme preparation was used The determination is described under a (Methods)

of bilirubin and of bilirubin monoglucuronide were studied separately Amounts of bilirubin glucuronidated and amounts of bilirubin monoglucuronide converted to diglucuronide increased linearly with time Velocities remained unchanged for 30 and 25 min, respectively These conditions are prerequisites for enzyme kinetic studies Velocities of bilirubin glucuronidation measured over the range 50-166 μM bilirubin fitted a straight line plotted double reciprocally according to LINEWEAVER-BURK[3] (Fig 3) Michaelis Menten constant (K_m) and maximal velocity (V_{max}) amounted to 9 6 μM and 13 3 nmoles/hr per mg protein In Fig 4 it is seen that velocities with which bilirubin diglucuronide was formed at different bilirubin monoglucuronide concentrations also fitted a straight line plotted according to LINEWEAVER-BURK K_m 13 8 μM, V_{max} 15 nmoles/hr per mg protein Measurements were made over the range 9 5-31 6 μM bilirubin monoglucuronide

Bilirubin glucuronidation was not inhibited by *p*-nitrophenol (Table II) The conversion of bilirubin monoglucuronide to diglucuronide, however, was found to be inhibited in the presence of *p*-nitrophenol A DIXON plot[3] of this inhibition shows it to be of the competitive type

TABLE II INFLUENCE OF *P*-NITROPHENOL ON THE GLUCURONIDATION OF BILIRUBIN

	B → BG+BG2 (nmoles/hr mg protein ± SD)	Number of experiments
Control	9 2 ± 0 1	4
+ 8 mM *p*-nitrophenol	9 4 ± 0 1	4

Reagent mixtures consisted of 200 μl bilirubin (50 μM), 200 μl UDPglucuronate (8 mM), 200 μl solubilized enzyme and 600 μl Tris-HCl buffer (0 05 M, pH 8 3) containing $MgCl_2$ (3 3 mM) *p*-Nitrophenol was added in a final concentration of 8 mM

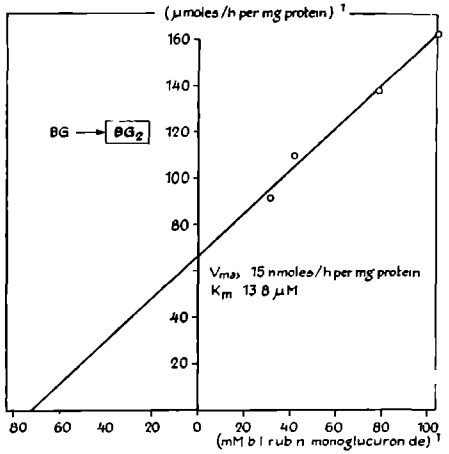

Fig 4 Glucuronidation of bilirubin monoglucuronide (BG→ $\boxed{BG_2}$) LINEWEAVER-BURK plot A solubilized enzyme preparation was used For details see legend of Table I

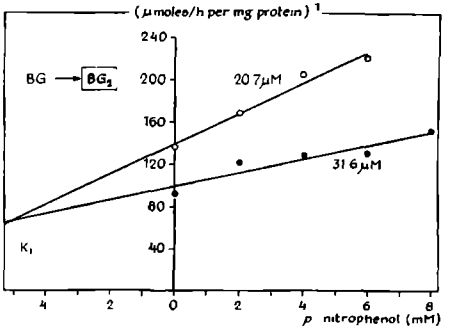

Fig 5 Influence of *p*-nitrophenol on the glucuronidation of bilirubin monoglucuronide (BG→ $\boxed{BG_2}$). DIXON plot[3] 1/reaction velocity is plotted against the inhibitor concentration Measurements were done at substrate concentrations of 20 7 μM and 31.6 μM bilirubin monoglucuronide UDPglucuronate concentration amounted to 5 mM A solubilized enzyme preparation was used For details see legend of Table I

(Fig 5) with an inhibitor constant (K_i) of 5 05 mM The K_m-value of *p*-nitrophenol glucuronidation found with this solubilized enzyme preparation from cat liver amounted to 4 80 mM (V_{max} 250 nmoles/hr per mg protein)

Discussion

Pre-treatment of microsomes with detergents such as Triton X-100 has been reported to activate glucuronidation processes[10] Detergents presumably affect penetration barriers, in the microsomal environment, between substrate and active site It was seen that Tween 80 can also act as an activating agent In addition to this, another effect of Tween 80 was observed Relatively less monoglucuronide and more diglucuronide was found after incubation of bilirubin with Tween-pretreated microsomes This effect of Tween shows that conversion of bilirubin mono- to diglucuronide should be distinguished from the conversion of bilirubin to bilirubin monoglucuronide When bilirubin monoglucuronide detaches from the site where it was formed and must penetrate to the site where mono- is converted to diglucuronide, Tween might exert its effect on penetration barriers in the vicinity of this second site Tween might also prevent that bilirubin monoglucuronide adheres to proteins and thus becomes unavailable as a substrate for the second step It was found that, when bilirubin was incubated with microsomes in the presence of albumin, relatively less diglucuronide and more monoglucuronide was formed In our incubation system, therefore, albumin at low concentration (9 mg/ml) did not influence the conjugation of bilirubin to monoglucuronide, whereas the conversion of mono- to diglucuronide was inhibited This adds further evidence that the conversion of bilirubin to bilirubin diglucuronide occurs in two distinct steps

The question whether the two glucuronidation steps are mediated by one or two enzymes was studied with a solubilized enzyme preparation. To study the two steps separately, both bilirubin and bilirubin monoglucuronide were used as substrates for glucuronidation After incubation of bilirubin with UDPglucuronate and enzyme, both mono- and diglucuronide were formed. Assuming that diglucuronide can only be formed via monoglucuronide it seems justified to regard the velocity with which bilirubin is glucuronidated as equal to the velocity of monoglucuronide for-

mation. The conversion of bilirubin to monoglucuronide and of bilirubin monoglucuronide to diglucuronide obeyed Michaelis Menten kinetics. K_m- and V_{max}-values for the two steps were found to be of the same order of magnitude. This is consistent with our finding that almost no bilirubin monoglucuronide was found in cat bile, whereas besides some other bilirubin conjugates, bilirubin diglucuronide was in excess[7].

In a previous study *p*-nitrophenol was shown to be glucuronidated with microsomes derived from cat liver[8]. In the present study we found that this compound inhibited the glucuronidation of bilirubin monoglucuronide competitively, whereas the conversion of bilirubin to monoglucuronide was not influenced. This is a strong indication that the two steps of bilirubin glucuronidation are mediated by two different enzymes. Since the K_i-value of *p*-nitrophenol for the glucuronidation of bilirubin monoglucuronide almost equals the K_m-value for the glucuronidation of *p*-nitrophenol itself, the bilirubin monoglucuronide-glucuronidating enzyme might also be active in the glucuronidation of *p*-nitrophenol.

Conclusion and clinical implications

1. It was found that for the cat the activities of the proposed first- and second-step enzymes (the enzymes mediating the glucuronidation of bilirubin and the glucuronidation of bilirubin monoglucuronide, E_1 and E_2, respectively) were of the same magnitude; hence for the cat $E_1/E_2 = 1$. In a previous study it was found that with microsomes derived from Wistar rat liver much more mono- than diglucuronide was formed, thus E_1/E_2 presumably > 1. It depends on this ratio whether or not, *in vivo*, bilirubin monoglucuronide is formed under special circumstances. The findings of OSTROW and MURPHY[11] that in bile of rats infused with bilirubin more mono- than diglucuronide was found, is consistent with our findings. A study on the E_1/E_2 ratio for human liver is in progress.

2. Glucuronidation of drugs might be mediated by either E_1 or E_2. When K_m-values for the glucuronidation of drugs are of the same order of magnitude as the K_m-value for *p*-nitrophenol glucuronidation, which appeared to be about 350 × that for bilirubin monoglucuronide glucuronidation, only large amounts of drugs can inhibit step 1 or step 2 of bilirubin glucuronidation. Bilirubin or bilirubin monoglucuronide, however, may be very effective inhibitors of glucuronidation of drugs by E_1 or E_2, respectively.

3. The Crigler-Najjar syndromes (chronic nonhaemolytic unconjugated hyperbilirubinaemia, with glucuronyl transferase deficiency) should be re-investigated. Since bilirubin is not glucuronidated by the liver of patients with this deficiency, they probably lack E_1. Whether they also lack E_2 is not yet known. In this respect it is interesting to know whether E_2 accepts bilirubin as substrate or not. According to ARIAS[1], there are two types of the Crigler-Najjar syndrome: Type I, severe jaundice often with signs of kernicterus, no bilirubin glucuronide in bile and not responding to phenobarbital treatment; and Type II, moderate jaundice, some bilirubin glucuronide in bile and responding to phenobarbital treatment. The modes of inheritance of types I and II are different: Type I, autosomal recessive and Type II, probably autosomal dominant. Possibly in Type I both E_1 and E_2, and in Type II only E_1 is lacking.

SUMMARY

Bilirubin glucuronidation was studied, *in vitro*, with cat liver microsomes. It was found that after incubation of bilirubin with microsomes pre-treated with Tween 80, relatively more diglucuronide and less monoglucuronide was formed than when incubations were done with untreated microsomes or with microsomes pre-treated with Triton X-100. When bilirubin was incubated with microsomes in the presence of a small amount of albumin, relatively less diglucuronide and more monoglucuronide was formed. From these results it was concluded that bilirubin is glucuronidated in two steps: first, bilirubin is conjugated with glucuronic acid to bilirubin monoglucuronide, and bilirubin monoglucuronide is subsequently converted to diglucuronide. *p*-Nitrophenol inhibited the second step competitively, whereas the first step was not influenced by this aglycone. Hence the conclusion seems valid that the two bilirubin glucuronidation steps are mediated by separate enzymes.

The Michaelis Menten constant (K_m) of *p*-nitrophenol glucuronidation and inhibitor constant (K_i) of *p*-nitrophenol for the glucuronida-

tion of bilirubin monoglucuronide were about equal. This means that bilirubin monoglucuronide-glucuronidating enzyme might also be active in the glucuronidation of *p*-nitrophenol.

Some clinical implications of these findings are discussed.

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REFERENCES

1. ARIAS, I. M., L. M. GARTNER, M. COHEN, J. BEN EZZER and A. J. LEVI (1969) Chronic nonhemolytic unconjugated hyperbilirubinemia with glucuronyl transferase deficiency. *Amer. J. Med.* **47**, 395.
2. BLACK, M., B. H. BILLING and K. P. M. HEIRWEGH (1970) Determination of bilirubin UDP-glucuronyltransferase activity in needle-biopsy specimens of human liver. *Clin. chim. Acta* **29**, 27.
3. DIXON, M. and E. C. WEBB (1964) *Enzymes*, 2nd edition. Longmans, London.
4. DUTTON, G. J. (1966) The biosynthesis of glucuronides, *in* G. J. DUTTON, *Glucuronic acid, free and combined*. Academic Press, New York, p. 186.
5. HEIRWEGH, K. P. M., G. P. VAN HEES, P. LEROY, F. P. VAN ROY and F. H. JANSEN (1970) Heterogeneity of bile pigment conjugates as revealed by chromatography of their ethyl anthranilate azopigments. *Biochem. J.* **120**, 877.
6. HENDERSON, P. TH. and K. J. KERSTEN (1970) Metabolism of drugs during rat liver regeneration. *Biochem. Pharmacol.* **19**, 2343.
7. JANSEN, P. L. M. (1972) Glucuronidation of bilirubin: a two-step reaction. In preparation.
8. JANSEN, P. L. M. and P. TH. HENDERSON (1972) The influence of phenobarbital treatment on *p*-nitrophenol and bilirubin glucuronidation in Wistar rat, Gunn rat and cat. *Biochem. Pharmacol*,, in press.
9. LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL (1951) Protein measurement with the folin phenol reagent. *J. biol. Chem.* **193**, 265.
10. MULDER, G. J. (1970) The effect of phenobarbital on the submicrosomal distribution of uridine diphosphate glucuronyltransferase from rat liver. *Biochem. J.* **117**, 319.
11. OSTROW, J. D. and N. H. MURPHY (1970) Isolation and properties of conjugated bilirubin from bile. *Biochem. J.* **120**, 311.
12. ROY, F. P. VAN and K. P. M. HEIRWEGH (1968) Determination of bilirubin glucuronide and assay of glucuronyltransferase with bilirubin as acceptor. *Biochem. J.* **107**, 507.

THE ISOMERISATION OF BILIRUBIN MONOGLUCURONIDE

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SUMMARY

In aqueous media bilirubin monoglucuronide is subject to isomerisation In this reaction two moles bilirubin monoglucuronide are converted into one mole bilirubin and one mole bilirubin diglucuronide Mainly the IIIα and IXα isomers of bilirubin were formed The tetrapyrrole moiety of bilirubin diglucuronide formed in this reaction had mainly the XIIIα and IXα structure Oxygen enhanced the reaction A free radical chain mechanism is the most likely process explaining our results

INTRODUCTION

The existence of bilirubin monoglucuronide as a chemical entity has been doubted for many years By running deproteinized serum of patients with liver disease through a silicone treated Kieselguhr column, Cole *et al* [1] obtained three bands, representing bilirubin and two more polar pigments The most polar one, Pigment II, consisted of bilirubin and glucuronic acid in a 1 2 molar ratio and was shown to be bilirubin diglucuronide[2] The other pigment, Pigment I, consisted of bilirubin and glucuronic acid in a 1 1 molar ratio and was therefore thought to be bilirubin monoglucuronide[3] The finding, however that on repeated chromatography this pigment dissociated into bilirubin and bilirubin diglucuronide led other authors to the conclusion that Pigment I is an equimolar complex of bilirubin and bilirubin diglucuronide rather than bilirubin monoglucuronide[3,4] Furthermore, by means of solvent partition at neutral pH, Nosslin[5] recovered unconjugated bilirubin from Pigment I Because of the uncertain nature of Pigment I and because of the lack of unequivocal analytical methods, there has never been much attention paid to the differentiation of bilirubin monoglucuronide and bilirubin diglucuronide in the diagnosis of liver disease, in spite of some promising earlier studies[6–8]

Recently, firm proof for the existence of bilirubin monoglucuronide was furnished[9–12] Bilirubin IXα is an asymmetrical molecule and its monoglucuronide derivative should therefore exist as two isomers, depending on which side of the molecule the glucuronyl group is attached These two isomers could be separated by means of TLC, after substitution of the conjugating group by NH_2, and their mass spectra were

analyzed[10]. In another study the structure of the azopigments, azodipyrrole and azodipyrrole-glucuronide, obtained by reaction of bilirubin monoglucuronide with the diazonium salt of *O*-ethyl anthranilate[11], was analyzed by means of mass spectrometry[12]. These studies showed that both isomers of bilirubin monoglucuronide occur in human and rat bile. McDonagh and Assisi[13] have shown that bilirubin IXα may isomerise in aqueous media, producing two symmetrical isomers, bilirubin IIIα and bilirubin XIIIα. We wish to report that also bilirubin monoglucuronide can undergo isomerisation, resulting in the production of bilirubin and bilirubin diglucuronide.

MATERIAL AND METHODS

Chemicals

Organic solvents were analytical reagent grade. Bilirubin, ascorbic acid, sodium nitrite, methyl propyl ketone, *n*-butyl acetate, chloroform and ethyl acetate were purchased from Merck A.G., Germany. Uridine-5'-diphosphoglucuronic acid (ammonium salt) was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Ammonium sulfamate was from Baker Chemicals N.V., The Netherlands. *o*-Ethylanthranilate was purchased from Eastman Organic Chemicals, Rochester, N.Y. and Triton X-100 from British Drug Houses Ltd., Poole, England. In thin-layer chromatography TLC-plates, Silica Gel 60, pre-coated, 20–20 cm, layer thickness 0.25 mm from Merck A.G., Germany, were used.

Preparation of bilirubin monoglucuronide

Bilirubin monoglucuronide was prepared enzymically. It is known that about 90% of the bilirubin glucuronides formed during incubation of bilirubin and UDP-glucuronate with a microsomal preparation from Wistar rat liver, consists of the monoglucuronide[11].

A microsomal preparation from Wistar rat liver was prepared as described previously[14]. Before incubation, Triton X-100 (0.25%, v/v) was added to this preparation. 4.4 mg bilirubin was dissolved in 0.5 ml NaOH (0.05 M) and this solution was diluted with NaH_2PO_4–Na_2HPO_4 buffer (0.1 M, pH 7.8). Incubation mixtures (48 ml) contained: bilirubin (83 μM), UDP-glucuronate (2.2 mM), $MgCl_2$ (3.3 mM), Tris–HCl buffer (0.1 M, pH 8.3) and the above described microsomal preparation. After incubation during 40 min at 37°, the pH was adjusted to pH 3.2–3.4 by addition of HCl (2 M) and bilirubin and the formed glucuronides were extracted with 25 ml ethyl acetate[9]. After evaporation of the ethyl acetate phase *in vacuo*, the compounds were dissolved in 25 ml Tris–HCl buffer (0.1 M, pH 8.3). This solution was thoroughly shaken with 25 ml chloroform in order to extract the unconjugated bilirubin. After this, the pH of the aqueous phase was adjusted to pH 3.2–3.4 and the bilirubin glucuronides were extracted with 25 ml ethyl acetate. Finally the ethyl acetate was evaporated *in vacuo* and the conjugates were dissolved in Tris-HCl buffer (0.05 M, pH 8.3).

Isomerisation of bilirubin monoglucuronide

1 ml of the monoglucuronide preparation was added to 9 ml NaH_2PO_4–Na_2HPO_4 buffer (0.1 M, pH 6.5) containing ethanol (5%, v/v). At certain time intervals 1 ml samples were removed for analysis. In addition a sample was removed from the monoglucuronide preparation for immediate analysis.

In order to study the reaction as a function of pH, mixtures consisting of 0 1 ml of the bilirubin monoglucuronide preparation, 0 05 ml ethanol and 1 ml buffer, were incubated for 45 min at room temperature The following buffers of varying pH were used NaH_2PO_4–Na_2HPO_4 buffer (0 1 M), Tris–HCl buffer (0 1 M) and glycine–NaOH buffer (0 1 M) Immediately after incubation diazo reagent A (see next paragraph), was added

During aerobic incubations the mixtures were kept in open vessels which were gently shaken by hand In order to incubate the solutions anaerobically, N_2 was passed through them The incubations were performed at 5 cm distance from an ordinary fluorescent lamp (Philips, TLD 15W/33)

Determination of bilirubin, bilirubin monoglucuronide and diglucuronide

The tetrapyrrole molecule of bilirubin and glucuronides is cleaved into two dipyrrole azopigments on reaction with the diazonium salt of *o* ethyl anthranilate[11] In aqueous media, at pH 2 8, only the conjugates of bilirubin react, but in organic media bilirubin also gives a positive diazo reaction This allowed us to differentiate between bilirubin and its glucuronides Thus, samples from the incubation mixtures were added respectively to Diazo reagent A, consisting of 4 ml glycine–HCl buffer (0 1 M, pH 2 8) and 2 ml diazonium solution[11], and Diazo-reagent B, consisting of 4 ml acetone–ethanol (1 1, v/v) and 2 ml diazonium solution[11] After incubation for 30 min at 25°, 1 ml ascorbic acid (1 g/10 ml) was added to stop the diazo reaction The azopigments were extracted with methyl propyl ketone–*n*-butyl acetate (17 3, v/v) From the solutions containing diazo B, the acetone–ethanol was evaporated and replaced by glycine buffer (0 1 M, pH 2 8) before extraction The extinction of the extracts was measured at 530 nm with a Beckman DB spectrophotometer The concentration of bilirubin and glucuronides was calculated by use of the previously determined extinction coefficient (ε_{530} 44 4 × 10^3 M^{-1} cm^{-1}) for azopigments in the above mentioned extraction medium[11]

In the diazo reaction one mole bilirubin diglucuronide gives rise to two moles azodipyrrole glucuronide One mole bilirubin monoglucuronide gives rise to one mole azodipyrrole and one mole azodipyrrole glucuronide in this reaction These two different azopigments can be separated by means of thin-layer chromatography Therefore samples from extract A were applied directly to pre-coated silicagel plates These plates were developed twice first in chloroform and subsequently in chloroform–methanol–water (65 25 3 v/v/v)[11] The spots representing azodipyrrole and azodipyrrole-glucuronide, were eluted in methanol The extinction of the eluates was determined spectrophotometrically at 530 nm From these extinctions the mono /diglucuronide ratio could be calculated[8]

Bilirubin isomers

The tetrapyrrole structure of bilirubin and its glucuronides was analyzed according to McDonagh and Assisi[15] Bilirubin glucuronides were hydrolyzed by addition of 1 ml NaOH (1 M) to the incubation mixtures A pinch of ascorbic acid was added to prevent oxidation After 30 min standing in the dark the solution was neutralized and the pigments were extracted with 4 ml chloroform A sample of this extract was applied immediately to a silicagel plate The plates were developed in chloroform–acetic acid (99 1, v/v) In order to determine the isomeric configuration of bilirubin, this com-

pound was extracted from the incubation mixture with chloroform at pH 8.3. Chromatography of this extract was performed as described above.

Commercial bilirubin is known to consist of bilirubin IXα with small amounts of bilirubin IIIα and XIIIα, and was therefore used as reference. The spots representing bilirubin IIIα, IXα and XIIIα (in order of decreasing R_f)[18], were eluted in chloroform–methanol (1:1, v/v). The extinction of the eluates was measured at 450 nm.

Abbreviations

B, BDG and BMG represent bilirubin, bilirubin diglucuronide and bilirubin monoglucuronide, respectively.

RESULTS

An aqueous solution, containing BMG in excess, was incubated at a short distance from an ordinary fluorescent lamp. In Table I it is seen that this resulted in a gradual decrease of the BMG concentration whereas the B and BDG concentration concomitantly increased. In experiment 1 the BMG concentration in the first 8 min decreased by 7.4 μM, whereas the concentrations of B and BDG rose by 3.8 μM and 3.7 μM, respectively. In the second 8 min period the BMG concentration decreased by 6.5 μM, whereas the rise of B and BDG amounted to only 1.5 μM and 1.8 μM. Experiment 3 in Table I shows a similar pattern. Incubation longer than 30 min resulted in a decreased recovery of all three compounds. In general it can be concluded from these experiments that about two moles BMG are converted into one mole B and one mole BDG. During prolonged incubation the part of the reaction leading to the formation of B and BDG becomes less complete until finally the rate of breakdown for B and BDG exceeds the rate of synthesis.

In experiment 1 of Table I, the total decrease in the BMG concentration amounted to 42%, which is about the same as the decrease in the first 15 min period in ex-

TABLE I

THE ISOMERISATION OF BMG AND THE FORMATION OF B AND BDG AT pH 6.5

Mixtures containing BMG in excess, were incubated for different periods of time. The amounts of B, BMG and BDG were determined before incubation and at different time intervals during incubation, as described in MATERIALS AND METHODS.

Incubation time (min)	E_{530}				*Concentrations (μM)*			*Recovery (%)*
	diazo B	*diazo A*	*azo-dipyrrole*	*a-glucuronide*	*B*	*BDG*	*BMG*	
1. Aerobically								
0	0.949	0.837	0.142	0.177	5.0	4.1	33.5	—
8	0.950	0.753	0.107	0.170	8.8	7.8	26.1	100
16	0.882	0.651	0.081	0.160	10.3	9.6	19.6	93
2. Anaerobically								
0	0.765	0.675	0.130	0.125	4.1	0	30.3	—
8	0.755	0.645	0.144	0.127	5.0	1.5	27.5	100
16	0.775	0.625	0.115	0.118	6.8	2.4	27.7	101
3. Aerobically								
0	0.585	0.490	0.094	0.170	4.3	6.3	15.7	—
15	0.570	0.410	0.056	0.161	7.3	8.9	9.5	97
30	0.550	0.340	0.031	0.148	9.5	10.0	5.3	94
45	0.505	0.300	0.024	0.138	9.2	9.5	4.0	87

periment 3 When the incubation was performed anaerobically, the reaction proceeded significantly slower after 16 min, the decrease in BMG amounted to only 9% Thus oxygen apparently plays a role in this reaction More vigorous oxygenation of the solution, however, when for example a magnetic stirrer at high speed was used, resulted in a decreased recovery of all three compounds

It depends on the hydrogen ion concentration whether or not the reaction can take place When a BMG solution was incubated at different pH's, the maximal amount of BDG was formed at pH 6 5 as is seen in Fig 1 Before incubation 13% of the mixture consisted of BDG This means that the BDG concentration in the mixtures with pH's below pH 5 2 or above pH 8 2 had actually decreased during the incubation, as can be derived from Fig 1

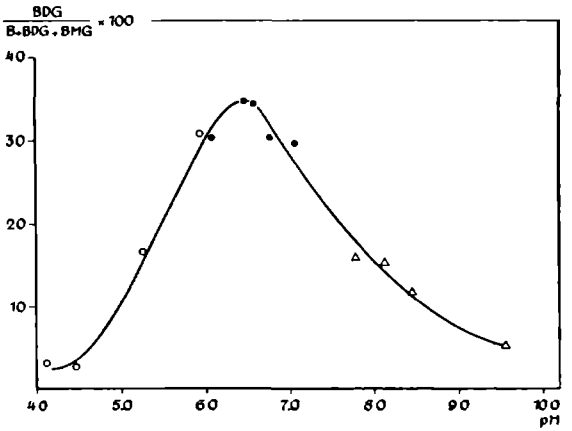

Fig 1 Isomerisation of BMG, as function of the pH Incubation mixtures containing 6 7 μM B, 3 7 μM BDG (13 3%) and 17 3 μM BMG were incubated for 45 min and analyzed as described in Material and Methods ○ Citrate–Na_2HPO_4 buffer (0 1 M) ● NaH_2PO_4–Na_2HPO_4 buffer (0 1 M) △, Tris–HCl buffer (0 1 M)

Analysis of the isomeric configuration of the bilirubin derivatives revealed that before incubation the tetrapyrrole with the asymmetrical IXα configuration predominated over the symmetrical IIIα and XIIIα isomers (Table II) After incubation, the IXα isomer had proportionally decreased and the IIIα and XIIIα isomers had increased Furthermore, it can be seen in Table II that mainly the IIIα and IXα isomers of B were formed Without risk of further isomerisation, BMG and BDG cannot be separated, therefore the tetrapyrrole structures of BMG and BDG could not be studied separately From the data depicted in Table II, however, it can easily be inferred that the formed BDG must consist mainly of B XIIIα DG and B IXα DG

From Table II it can be deduced that all three BMG isomers, B IIIα MG, B IXα MG and B XIIIα MG, have taken part in the reaction Before incubation the mixture contained 2 8 μM B IIIα MG, 50 5 μM B IXα MG and 13 0 μM B XIIIα MG After incubation the total concentration of BMG amounted to 10 2 μM This means that the concentration of both B IXα MG and B XIIIα MG had decreased It can be calculated that the B IIIα MG concentration after incubation amounted to maximally

TABLE II

ANALYSIS OF THE TETRAPYRROLE STRUCTURE OF B, BMG AND BDG, BEFORE AND AFTER INCUBATION AT pH 6 5

A mixture containing BMG in excess, was incubated aerobically for 45 min. The amounts of B, BMG and BDG were determined before and after incubation The tetrapyrrole structure of B, BMG and BDG was determined after hydrolysis of the glucuronides In order to determine the isomeric configuration of B, this compound was extracted from the mixture with chloroform, as described in MATERIAL AND METHODS

Time (min)	*Constituents*	*Tetrapyrrole isomers (%)*			*Concentrations (μM)*		
		IIIα	*IXα*	*XIIIα*	*B*	*BDG*	*BMG*
0	B + BDG + BMG	10 2	72 6	17 2	9 2	0	66 3
	B	53 3	46 7	0			
	BDG + BMG	4 2	76 2	19 6			
45	B + BDG + BMG	28 4	42 0	29 6	39 4	21 9	10 2
	B	51 0	43 2	5 8			
	BDG + BMG	0 6	40 5	58 9			

0.2 μM. Thus besides B IXα MG and B XIIIα MG, also B IIIα MG must have taken part in the reaction.

DISCUSSION

Bilirubin and its conjugates react with the diazonium salt of *o*-ethyl anthranilate to form stable azopigments which can be quantitatively analyzed on TLC[11]. In aqueous media, at pH 2.8 (diazo A), only conjugated bilirubin reacts with this reagent[11], whereas in an acetone–ethanol medium (diazo B) both conjugated and unconjugated bilirubin give a positive reaction[16].

In the present study a solution containing BMG in excess, was incubated and at different time intervals samples were drawn from it and submitted to both diazo reactions. As a result of the incubation a significant and steady decrease of the products giving only a positive reaction with diazo A, was found. This is evidently the result of a reduction of the concentration of either BDG or BMG. Concomitant with this, an increased azodipyrrole-glucuronide/azodipyrrole ratio was found. Calculation of the absolute BMG and BDG concentrations revealed that BDG had increased and BMG decreased during incubation.

Furthermore it was found that during incubation the products giving only a positive reaction with diazo B, had increased. This must be the result of a rise in the concentration of B as other possible reaction products, like pentdyopent pigments, do not react with diazo A or diazo B, or, as for example hydroxypyrromethenecarbinol, also give a positive reaction with diazo A[17].

In addition it was found that the tetrapyrrole moiety of the compounds did not keep the normal asymmetrical IXα configuration. This inevitably leads to the conclusion that our findings are the result of isomerisation of BMG. Under ideal circumstances two moles BMG were converted into one mole B and one mole BDG, as a result of this process. Although the reverse reaction, the formation of BMG from B and BDG, was not studied in detail, there were some indications that this may occur below pH 5.2 or above pH 8.2. As the underlying mechanism for the observed reaction a free radical chain reaction is the most probable. The several possibilities of such a process are depicted in Fig. 2. The scheme in Fig. 2 has been set up for a solution only

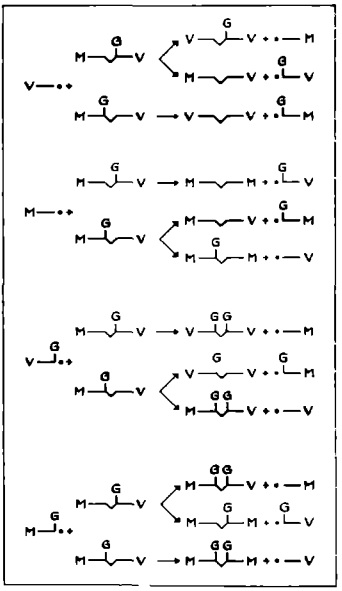

Fig 2 Isomerisation of BMG by means of a free radical mechanism The propagation of the reaction

M—(G)—V isovinyl isomer of B IXa MG

M—(G)—V vinyl isomer of B IXα MG

V—V M—V M—M represent B IIIα B IXα and B XIIIα respectively The mono and diglucuronide derivatives of these bilirubin isomers are represented by means of attachment of one or two glucuronyl groups ($\overset{G}{|}$) to these structures

V— M— V—(G) M—(G) represent free radicals with a dipyrrole or a dipyrrole methylene structure

containing B IXα MG The heavy printed pathways are those which give rise to the compounds found in the analysis of Table II It is apparent that the vinyl isomer of B IXα MG is more susceptible to isomerisation than the isovinyl isomer The radicals depicted in the scheme can be either dipyrrole or dipyrrole methylene fragments An important determinant for the reaction seems to be the hydrogen ion concentration of the medium Oxygen and light apparently have a catalytic effect on this process In particular oxygen may play a role in the initiation of the reaction This, however, warrants further study

In older communications it has been reported that the so called Pigment I from the serum of patients with liver disease on repeated chromatography dissociated into B and BDG[3,4] This chromatography has been performed at pH 6 (ref 3) at which pH BMG easily isomerises into B and BDG Thus possibly Pigment I is BMG instead of a complex of B and BDG This evokes several questions Is BMG an important

compound in the serum of patients with obstructive icterus? Is BMG a toxic compound as it may be the origin of radicals which may attach to tissue compounds as enzymes and DNA? This may in particular play a role in the skin under the influence of light. Furthermore the question has to be reconsidered whether or not differentiation of BMG and BDG by the determination of conjugated bilirubin holds promises for the diagnosis of liver disease. By development of methods for this, one has to reckon with the findings reported in the present communication.

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REFERENCES

1 P G Cole, G H Lathe and B H Billing, *Biochem J*, 57 (1954) 514
2 B H Billing, P G Cole and G H Lathe, *Biochem J*, 65 (1957) 774
3 C H Gregory, *J Lab Clin Med*, 61 (1963) 917
4 A Ph Weber, L Schalm and J Witmans, *Acta Med Scand*, 173 (1963) 19
5 B Nosslin, *Scand J Clin Lab Invest*, 12 (1960) suppl 49
6 N H Hoffman, F F Whitcomb, H R Butt and J L Bollman, *J Clin Invest*, 39 (1960) 132
7 L J Schoenfield, J L Bollman and N H Hoffman, *J Clin Invest*, 41 (1962) 133
8 L J Schoenfield and J L Bollman, *Proc Soc Exp Biol Med*, 112, (1963) 929
9 J D Ostrow and N H Murphy, *Biochem J*, 120 (1970) 311
10 F H Jansen and B H Billing, *Biochem J*, 125 (1971) 917
11 F P Van Roy and K P M Heirwegh, *Biochem J*, 107 (1968) 507
12 F H Jansen and M S Stoll, *Biochem J*, 125 (1971) 585
13 A I McDonagh and F Assisi, *Biochem J*, 129 (1972) 797
14 P L M Jansen and P Th Henderson, *Biochem Pharmacol*, 21 (1972) 2457
15 A F McDonagh and F Assisi, *FEBS Letters*, 18 (1971) 315
16 F P Van Roy, J A T P Meuwissen, F De Meuter and K P M Heirwegh, *Clin Chim. Acta*, 31 (1971) 109
17 T K With, *Biologie der Gallenfarbstoffe*, Thieme Verlag, Stuttgart, 1960, p 31

THE ENZYME-CATALYZED FORMATION OF BILIRUBIN DIGLUCURONIDE BY A SOLUBILIZED PREPARATION FROM CAT LIVER MICROSOMES

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Summary

When bilirubin monoglucuronide is incubated with a preparation from the 105 000 × *g*-supernatant of deoxycholate-treated cat liver microsomes, bilirubin diglucuronide is formed. This is an UDPglucuronate-dependent reaction whereby bilirubin IXα monoglucuronide is stoichiometrically converted into bilirubin IXα diglucuronide.

The pH optimum for the conversion of bilirubin into bilirubin monoglucuronide lies between pH 8.0 and pH 8.8. For the conversion of mono- into diglucuronide two optima were found, one at about pH 6.5 and another at pH 8.1.

When the incubation was performed at pH 6.5 and the enzyme protein concentration was lowered, bilirubin monoglucuronide started to isomerise. As a result of this isomerisation bilirubin diglucuronide is also formed. Diglucuronide formation according to this mechanism however, can be clearly differentiated from the enzyme-catalyzed diglucuronide formation.

By the formation of bilirubin monoglucuronide, one monoglucuronide isomer is preferentially synthesized.

The alkaline-labile bilirubin conjugates in the bile of cats and rats have mainly the IXα isomeric structure. This suggests that in these animals bilirubin diglucuronide is formed enzymically as the bilirubin moiety of diglucuronide, formed by means of the isomerisation reaction, has predominantly the XIIIα structure.

Introduction

Before excretion in the bile, bilirubin is conjugated with glucuronic acid, glucose or xylose [1,2]. Furthermore bilirubin may be conjugated with disaccharides [3]. Bilirubin diglucuronide however, is the most important conjugate in normal mammalian bile [1,2].

The question as to how this diglucuronide is formed in the liver has not yet been fully answered It has been reported that both bilirubin monoglucuronide and diglucuronide are formed when bilirubin and UDPglucuronate are incubated with homogenates from rat [4,5] or cat [6] livers. The monoglucuronide/diglucuronide ratio is influenced by several factors. For rats, strain differences seem to play a role Fevery et al. [4] reported the formation of predominantly the monoglucuronide with homogenates from Wistar rat livers. In contrast Halac et al. [5] found that with preparations from the rough microsomal fraction from the livers of Fisher strain rats, the diglucuronide was formed in excess But after storage of this preparation for 72 h, almost only bilirubin monoglucuronide was formed. In our laboratory it was observed that incubation with preparations from cat liver yielded about equal amounts of mono- and diglucuronide [6]. Pretreatment of these preparations with Tween 80 caused a shift in their ratio towards the latter compound. These results suggest that the conversion of bilirubin into its diglucuronide occurs in two distinct steps, with the monoglucuronide as an intermediate

In the first step glucuronic acid is attached to a carboxyl group of bilirubin. This must be an enzyme-catalyzed reaction as it is well known that the glucuronidation of unconjugated bilirubin requires the presence of UDPglucuronate and homogenates or microsomal preparations from liver, kidney or intestinal tissue [7] Only for N-glucuronides it is known that they may be synthesized in a non-enzymic way [7]. Recently it was found that bilirubin diglucuronide may also be formed non-enzymically, by means of isomerisation of bilirubin monoglucuronide [8] In this reaction 2 moles monoglucuronide are converted into 1 mole of unconjugated bilirubin and 1 mole of diglucuronide. This only occurred between pH 5 2 and pH 8 2, with an optimum at pH 6.5. Whether this mechanism plays a role by diglucuronide formation in the liver or does only occur, in vitro, in protein-free media, has not been studied yet Possibly, diglucuronide formation in the liver occurs by means of an enzyme-catalyzed transfer of glucuronic acid from UDPglucuronate to the free carboxyl of bilirubin monoglucuronide The present study was undertaken to elucidate this.

Materials and Methods

Animals

Cats were 12 - 18 months and Wistar rats 3 months old. Only male animals were used. Before killing the animals were fasted for 12 h.

Chemicals

Bilirubin, L(+)ascorbic acid, sodium nitrite, EDTA (disodium salt), sucrose, tris(hydroxymethyl)aminomethane, mono- and dibasic sodium phos-

phate, glycine, citric acid, $MgCl_2$ and the organic solvents methyl propyl ketone, *n*-butyl acetate and ethyl acetate were purchased from Merck A G, Darmstadt, Germany. Uridine 5'-diphosphoglucuronic acid (ammonium salt) and 7-deoxycholic acid (sodium salt) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Ammonium sulfamate was from J.T. Baker Chemicals N.V., Deventer, The Netherlands Glucaro-1,4-lactone was from Calbiochem, San Diego, Calif, U.S.A. *o*-Ethylanthranilate was purchased from Eastman Organic Chemicals, Rochester, N.Y., U.S.A. and Triton X-100 from British Drug Houses Ltd, Poole, England Sephadex G-25, coarse, was from Pharmacia Fine Chemicals A B., Uppsala, Sweden.

In thin-layer chromatography plates (glass), Silica Gel 60, precoated, 20 cm × 20 cm, layer thickness 0.25 mm from Merck A.G., Darmstadt, Germany, were used.

Tris buffer was prepared by adding 0.2 HCl to a 0.2 M Tris solution until the desired pH and this solution was diluted with distilled water until a final Tris concentration of 0 05 M or 0 1 M. Glycine—NaOH and glycine—HCl buffer was made by the addition of 0.2 M NaOH or 0.2 M HCl, respectively, to a 0 2 M glycine solution. After the desired pH was reached, the solutions were diluted to a glycine concentration of 0.1 M, phosphate buffer was made by mixing solutions of 0.1 M monobasic phosphate and 0.1 M dibasic phosphate until the desired pH. Citrate—phosphate buffer was made by mixing solutions of 0.1 M citric acid and 0.2 M dibasic phosphate until the desired pH.

Equipment

Cat liver was homogenized with an Ultra-Turrax apparatus from Janke und Kunkel K G., Staufen i Br., Germany, and rat liver with a Teflon-glass Potter—Elvehjem homogenizer from Tri-R Instruments Rockville Centre, N.Y., U.S.A. The operation speed of these apparatuses was 8000 and 5000 rev./min, respectively

Low speed centrifugation was done with a Heraeus—Christ Type UJ3 centrifuge For centrifugation at 9000 × *g* an I.E C. B-20 centrifuge was used. A Christ Omega LI ultracentrifuge was used for centrifugations at 105 000 × *g*

Spectrophotometrical determinations were done with a Beckmannn DB spectrophotometer. For the scanning of chromatograms a Zeiss PMQ II spectrophotometer, equipped with a scanning apparatus, was used. pH measurements were done with a Radiometer Model TTT2 pH meter, provided with a combination electrode.

Microsomal preparations

20% (w/v) homogenates were prepared from cat or rat liver in Tris buffer (0.05 M, pH 7 8), containing sucrose (0.25 M) and EDTA (sodium salt) (0.01 M). The homogenates were centrifuged at 9000 × *g*, for 20 min at 2°C. These supernatants were centrifuged at 105 000 × *g* for 1 h at 2°C. The microsomal fraction, thus obtained, was suspended in the homogenization medium. 1 ml of suspension contained microsomes derived from 1 g of liver.

Solubilized fractions from cat liver microsomal preparations

To the microsomal suspension from cat liver, solid deoxycholate (0.3%,

w/v) was added. After stirring for 4 h at 4°C with a magnetic stirrer, the suspensions were centrifuged at 105 000 × *g* for 1 h. The clear phase of the supernatant was passed over a column (16 mm × 1000 mm) containing Sephadex G-25, coarse. As an elution fluid Tris buffer (0.05 M, pH 7.8), containing KCl (0.14 M) and EDTA (0.01 M), was employed This procedure is a modification of the method described by Halac and Reff [9]. After it was found that a more active preparation was obtained with use of a larger Sephadex column (30 mm × 1000 mm), the above mentioned column was replaced by the latter.

Glucuronidation of bilirubin

Bilirubin (4.4 mg) was dissolved in 0.5 ml 0.05 M NaOH and this solution was diluted with phosphate buffer pH 7.8 (1 : 30, v/v). Incubation mixtures consisted of 0.2 ml of the bilirubin solution (final concn 83 μM), 0.2 ml solubilized enzyme preparation from cat liver (final protein concn 1.5 mg/ml) and 0.8 ml Tris buffer (0.1 M, pH 8.3) containing UDPglucuronate, $MgCl_2$ and glucuro-1,4-lactone (5 mM, 3.3 mM and 1 mM, respectively; all final concns). When the reaction was studied as function of pH, the pH of the Tris buffer was varied or the Tris buffer was replaced by phosphate or glycine — NaOH buffer of varying pH. After incubation for 30 min at 37°C in a shaking water bath, the mixtures were submitted to the diazo reaction described by Van Roy and Heirwegh [10]. After reaction with the diazonium salt of *o*-ethylanthranilate, in an aqueous medium at pH 2.8, the azo pigments were extracted with methylpropyl ketone—*n* butyl acetate (17 · 3, v/v) and the colour of this extract was determined spectrophotometrically at 530 nm. Conjugated bilirubin was calculated assuming ϵ_{530} 44.4 × $10^3 \cdot M^{-1} \cdot cm^{-1}$ (ref. 10)

In the diazo reaction the tetrapyrrole molecule of conjugated bilirubin is cleaved into dipyrrole azo pigments. One mole bilirubin diglucuronide gives rise to two moles azodipyrrole glucuronide and one mole bilirubin monoglucuronide to one mole azodipyrrole and one mole azodipyrrole glucuronide The two different azo pigments can be separated by means of thin-layer chromatography and from their ratio the amounts of mono- and diglucuronide can be calculated (formula for calculation is given in refs 11 and 6) Thus, samples of the azo pigment extract were applied directly on silica gel plates and the chromatograms were developed first in chloroform and subsequently in chloroform—methanol—water (65 : 25 · 3, v/v/v) [4]. The two clearly defined spots, representing the azo pigments, were eluted in methanol and their extinction was measured at 530 nm.

Preparation of bilirubin monoglucuronide

Bilirubin monoglucuronide was prepared biosynthetically. 0 02 ml of Triton X-100 was dissolved in 8 ml of rat liver microsomal preparation. The incubation mixtures consisted of this solution and 8 ml of bilirubin solution (prepared as described in the previous paragraph) together with 32 ml of Tris buffer (0.1 M, pH 8.3) containing UDPglucuronate and $MgCl_2$ (2.2 mM and 3 3 mM, respectively, final concns). After incubation for 40 min at 37°C, the pH was adjusted to pH 3.2 with 2 M HCl. Bilirubin and the formed glucuronides were extracted with 25 ml ethyl acetate [11]. After evaporation of the ethyl acetate in vacuo, the residue was dissolved in 25 ml Tris buffer (0.1 M,

pH 8.3). From this solution, unconjugated bilirubin was partly extracted with chloroform. The pH of the aqueous phase was adjusted tc pH 3.2 and the bilirubin glucuronides were extracted with 25 ml ethyl acetate. Finally the ethyl acetate phase was evaporated in vacuo and the residue was dissolved in Tris buffer (0.05 M, pH 8 3).After its preparation, the monoglucuronide solution was used without delay.

Glucuronidation of bilirubin monoglucuronide

The incubation mixture consisted of 0.2 ml of monoglucuronide solution (concn specified in tables and figures), 0.2 ml of solubilized enzyme preparation from cat liver and 0 8 ml of phosphate buffer (0.1 M, pH 6.5) containing UDPglucuronate, $MgCl_2$ and glucaro-1,4-lactone (3 mM, 3.3 mM and 1 mM, respectively, final concns). In a part of the study the pH of the phosphate buffer was varied or this buffer was replaced by citrate—phosphate buffer, Tris buffer or a glycine — NaOH buffer of varying pH. The mixtures were made in duplicate. One was submitted to the diazo reaction after incubation for 30 min at 37°C, and in the other one the diazo reaction was immediately started, without prior incubation. In this way the mono- and diglucuronide concentrations, after and before incubation, could be determined.

In experiments where besides the mono- and diglucuronide concentration also the concentration of unconjugated bilirubin was determined, the volume of the above described incubation mixture was doubled. An aliquot of 1.2 ml, taken from the incubated anf from the non-incubated tube, was processed as described above. To an equal aliquot 4 ml of acetone—ethanol (1 : 1, v/v) and 2 ml of diazo reagent was added. The diazo reaction was allowed to proceed for 30 min at 25°C and was stopped by adding 0.5 ml of ascorbic acid (1 g/10 ml). Acetone · ethanol was evaporated in vacuo, and 4 ml of glycine — HCl buffer (0.1 M, pH 2.8) was added. The azo pigments were extracted with methylpropyl propyl ketone—*n*-butyl acetate (17 . 3, v/v), the extinction was measured at 530 nM and the amounts of bilirubin plus glucuronides were calculated. The glucuronide concentration was subtracted from this value to yield the amount of unconjugated bilirubin.

Bilirubin isomers

Bilirubin glucuronides were hydrolyzed by the addition of 1 ml 1M NaOH (final pH 12.3) to the incubation mixture. A pinch of ascorbic acid was added to prevent oxidation. After 30 min the solution was neutralized (pH 7.4) and the pigments were extracted with 4 ml of chloroform. A sample of this extract was immediately applied to a silica gel plate The plates were dryed with N_2 and developed without delay in chloroform—acetic acid (99 .1, v/v) [12]. The spots representing the bilirubin isomers IIIα, IXα and XIIIα [12], were eluted in chloroform—methanol (1 · 1, v/v). As reference standard, a chloroform extract from the previously described bilirubin solution was used. The chromatogram of this extract revealed besides an abundant amount of bilirubin IXα also two faint spots representing bilirubin IIIα and XIIIα. The extinctions were measured at 450 nm.

Bile

Bile from rats was collected during 10 h after cannulation of their bile ducts. Cat bile was obtained by aspiration from the gall bladder, after killing the animal. Bile samples were submitted to the diazo reaction at pH 2.8 and chromatograms were developed according to Fevery et al. [4].

For determination of the bilirubin isomers in bile, the glucuronides were hydrolyzed and the isomeric composition was determined by means of thin-layer chromatography, exactly as described in the previous paragraph. The chromatograms were scanned at 450 nm.

Azodipyrrole and azodipyrrole glucuronide vinyl—isovinyl isomerism

The vinyl and isovinyl isomer of azodipyrrole and azodipyrrole glucuronide were determined as described by Jansen and Stoll [13].

Protein determination

This was done as described by Lowry et al. [14].

Results

Bilirubin and bilirubin monoglucuronide were incubated at different pH values with UDPglucuronate and the solubilized fraction from cat liver microsomes. In Fig. 1 it is seen that the pH optimum for the glucuronidation of unconjugated bilirubin lies between pH 8.0 and pH 8.8. For the conversion of

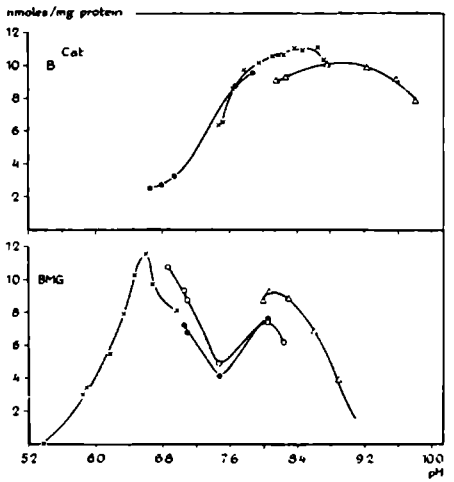

Fig 1. The conversion of bilirubin and bilirubin monoglucuronide as function of pH. Upper panel: bilirubin (83 μM) was incubated with UDPglucuronate (5 mM) and a solubilized preparation from cat liver for 30 min at 37°C. (●) Phosphate buffer (0.1 M); (X) Tris buffer (0.1 M), (^) Glycine—NaOH buffer (0.1 M) Lower panel: Bilirubin monoglucuronide was incubated with UDPglucuronate (3 mM) and a solubilized preparation from cat liver for 30 min at 37°C. (X) Citrate—phosphate buffer (0.1 M), substrate concn 22 μM monoglucuronide; (○) phosphate buffer (0.1 M), 24 μM monoglucuronide; (●) phosphate buffer (0.1 M), 16μM monoglucuronide; (^) glycine-NaOH buffer (0.1 M), 17 μM monoglucuronide. The ordinate represents the amount of substrate converted in 30 min.

TABLF I

Incubation of bilirubin monoglucuronide at pH 8 1 and pH 6 5 in the presence and in the absence of an enzyme preparation from cat liver The incubations were performed for 0 and 30 min at 37° C The mixtures 1 and 4 were protein-free whereas the mixtures 2, 3, 5, 6, 7 and 8 contained a solubilized enzyme preparation from cat liver UDPglucuronate (UDPGA) (3 mM) was added to Mixtures 3 and 6, UDP (9 mM) to Mixtures 7 and 8 B, BMG and BDG are expressed as nmoles/ml incubation medium, and represent bilirubin monoglucuronide and diglucuronide, respectively

Conditions	Time	B	BMG	BDG	Recovery (%)
1 pH8 1 - E — UDPGA	0	12 4	19 6	2 2	—
	30	8 6	25 4	0	100
2 pH8 1 + E — UDPGA	0	12 4	19 6	2 2	—
	30	11 7	20 5	1 3	98
3 pH8 1 + E + UDPGA	0	7 5	18 2	3 4	—
	30	8 9	2 0	19 3	104
4 pH6 5 — E — UDPGA	0	5 0	17 0	4 8	—
	30	8 6	7 6	9 6	96
5 pH6 5 + E — UDPGA	0	13 9	14 9	1 0	—
	30	17 0	18 9	2 1	127
6 pH6 5 + E + UDPGA	0	7 5	18 2	3 4	—
	30	9 0	2 0	17 1	97
7 pH8 1 + E + UDP	0	12 4	19 6	2 2	—
	30	9 8	17 5	2 0	86
8 pH6 5 + E + UDP	0	13 9	14 9	1 0	—
	30	13 9	12 5	2 7	98

mono- into diglucuronide two optima were found One at pH 8.1 and another one at pH 6 5

Incubation of bilirubin monoglucuronide at pH 6 5, in the absence of enzyme protein, results in its isomerisation (Table I, Reaction 4). At pH 8.1, a reverse reaction takes place under these circumstances
(1 bilirubin + 1 diglucuronide → 2 monoglucuronide, Reaction 1),
confirming our earlier findings [8] Both these reactions are inhibited in the presence of enzyme protein (Reactions 2 and 5) After addition of enzyme protein, diglucuronide only occurs in the presence of UDPglucuronate. This was found at both pH 6 5 and pH 8.1 (Reactions 3 and 6).

Table II shows that by diglucuronide formation by means of isomerisation of bilirubin monoglucuronide, a rearrangement of the tetrapyrrole moiety occurs. This, obviously, did not occur by the formation of diglucuronide in the presence of UDPglucuronate and enzyme protein. The data of Tables I and II show that this latter reaction occurs according to the equation
1 bilirubin IXα monoglucuronide → 1 bilirubin IXα diglucuronide
It has been reported that the glucuronidation of *p*-nitrophenol can also occur in the reverse direction [15]. In order to study whether this also holds true for the glucuronidation of bilirubin, monoglucuronide was incubated with UDP and a solubilized preparation from cat liver This, however, did not result in the formation of bilirubin, as is shown in Table I (7 and 8).

When unconjugated bilirubin is used as substrate and the incubation is performed at pH 8.1, both mono- and diglucuronide are formed. This is obviously the consequence of the finding that at pH 8.1 both glucuronidation steps can take place In Fig. 2 the amounts of mono- and diglucuronide, present at the end of the incubation, are depicted. The sum of these amounts is equal to

TABLE II

The isomeric configuration of the bilirubin moiety by the spontaneous and the enzyme-catalyzed formation of bilirubin diglucuronide. Mixtures containing bilirubin monoglucuronide were incubated and the diglucuronide concentration before and after incubation was determined. The percentage diglucuronide [diglucuronide/(bilirubin, mono-+diglucuronide) × 100] is represented in the table. Mixtures 1 and 2 were incubated in the presence of a solubilized preparation from cat liver and UDPglucuronate (3 mM) for 0 and 30 min at 37°C. Mixture 3 was protein-free and was incubated for 0 and 45 min at room temperature. For analysis of the tetrapyrrole structure of bilirubin and its glucuronides, the mixture was treated with NaOH and analyzed as described in Material and Methods.

Mixtures	Conditions	Time	BDG(%)	Tetrapyrrole isomers (%)		
				IIIα	IXα	XIIIα
1	pH 8.1 + E + UDPGA	0	12	13	63	24
		30	63	11	69	20
2	pH 6.5 + E + UDPGA	0	12	13	63	24
		30	61	13	62	25
3	pH 6.5 — E — UDPGA	0	0	10	73	17
		45	31	28	42	30

the total amount of bilirubin converted into monoglucuronide during the reaction. In Fig. 2 it is seen that coupling of the first glucuronic acid molecule to bilirubin is a linear function of time. After a certain lag period also the conversion of mono- into diglucuronide is a linear function of time and proceeds at the same rate as the first step.

With bilirubin monoglucuronide as substrate some characteristics of the second glucuronidation step were studied. In Fig. 3 it is seen that the conversion of mono- into diglucuronide is a linear function of time at both pH 6.5 and pH 8.1. In Fig. 4a it is shown that the non-UDPglucuronate-dependent conversion of mono- into diglucuronide by means of the isomerisation reaction

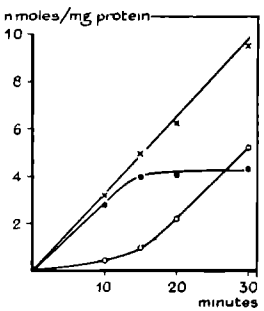

Fig. 2. The formation of bilirubin mono- and diglucuronide during incubation of bilirubin, as function of time. Bilirubin (83 μM) was incubated with UDPglucuronate (5 mM) and a solubilized preparation from cat liver, for varying periods of time at 37°C. After incubation the mixtures were submitted to the diazo reaction as described in Materials and Methods. The azo pigments were extracted with methylpropyl ketone—*n*-butyl acetate (17/3, v/v). The extinction of these extracts was determined at 530 nm. This allowed the calculation of the total amount of bilirubin conjugated. Aliquots of the extracts were applied to silica gel plates. After development of the chromatograms the spots, representing azodipyrrole and azodipyrrole—glucuronide, were eluted and the extinction of the eluates was determined at 530 nm. On the basis of these data, the amounts of bilirubin mono- and diglucuronide could be calculated. ×, total amount of bilirubin conjugated; •, bilirubin monoglucuronide; ○, diglucuronide. Note, in the experiments, depicted in Figs. 1 and 2, the smaller type Sephadex column (16 mm × 1000 mm) was used, by the preparation of the enzyme preparation. In all other experiments, deoxycholate was washed from the enzyme preparation with a larger Sephadex column (30 mm × 1000 mm).

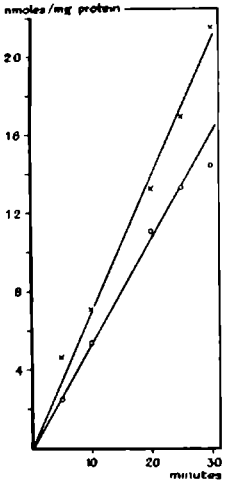

Fig. 3. The conversion of bilirubin monoglucuronide into diglucuronide as function of time. x, bilirubin monoglucuronide (19 μM) was incubated with 3 mM UDPglucuronate and a solubilized enzyme preparation from cat liver for varying periods of time at pH 8.1. ○, 13 μM monoglucuronide was incubated at pH 6 5 under the same conditions The ordinate represents the amount of diglucuronide, produced at the different time intervals, in nmoles/mg protein The incubation time is depicted on the abscissa

becomes more important at lower enzyme protein concentrations. This causes the curve, representing the formation of diglucuronide at pH 6.5 as function of the enzyme protein concentration not to go through zero. The calculated curve, however, representing the UDPglucuronate-dependent reaction, did not show this deviation and resembled the curve obtained at pH 8.1 (Fig. 4b).

The UDPglucuronate dependence of the enzyme-catalyzed bilirubin diglucuronide formation at pH 8.1 and 6.5 is shown in Fig. 5 by means of a Line-

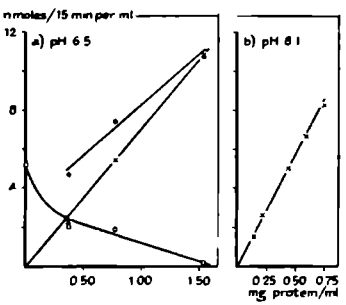

Fig. 4. The formation of bilirubin diglucuronide as a function of the protein concentration. In Fig. 4a bilirubin monoglucuronide (24 μM) was incubated at pH 6.5, with (●) and without (○) UDPglucuronate (3 mM) and a solubilized preparation from cat liver The concentration of the enzyme preparation was varied. Line (X) was obtained through subtraction of the lower from the upper line. In Fig. 4b monoglucuronide (19 μM) was incubated at pH 8 1 under the same conditions. The abscissa represents the final protein concentration and on the ordinate the amount of diglucuronide, produced in 15 min/ml incubation medium, is depicted.

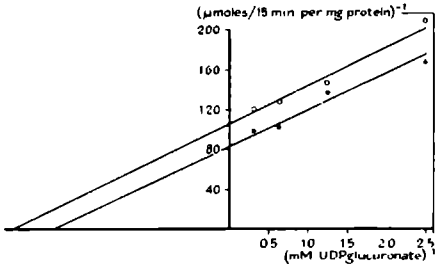

Fig 5 The glucuronidation of bilirubin monoglucuronide as a function of UDPglucuronate Lineweaver–Burk plot UDPglucuronate, in varying concentrations and solubilized enzyme preparation from cat liver were incubated with 16 μM bilirubin monoglucuronide at pH 8 1 (●) or with 17 μM bilirubin monoglucuronide at pH 6 5 ( ) for 15 min at 37°C The reciprocal values for the initial velocities, obtained at different UDPglucuronate concentrations are plotted on the ordinate

weaver-Burk plot. From these plots the following kinetic constants can be derived: K_{UDPGA} 0.44 mM, V 12 0 nmoles/15 min per mg protein at pH 8.1 by a fixed bilirubin monoglucuronide concentration of 16 μM, and K_{UDPGA} 0.36 mM, V 9 4 nmoles/15 min per mg protein at pH 6 5 by a fixed bilirubin monoglucuronide concentration of 17 μM.

Site of attachment of the glucuronyl group

Bilirubin IXα, the natural heme breakdown product, is an asymmetrical molecule. Therefore, two bilirubin IXα monoglucuronide isomers could exist, depending on the site of attachment of the glucuronyl group (either R_1 or R_2 in Fig. 6). Proof for the existence of these two isomers has been given by Jansen and Billing [16]. The two isomers can be distinguished by means of analysis of their azo pigments [13]. When, in Fig. 6, R_1 = glucuronic acid, equimolar amounts of vinyl azodipyrrole glucuronide and isovinyl azodipyrrole are derived from the compound in the diazo reaction, when R_2 = glucuronic acid, equimolar amounts of isovinyl azodipyrrole-glucuronide and vinyl azodipyrrole are derived from it From Table III it can be derived that the glucuronyl group is preferentially attached to R_1, when unconjugated bilirubin is incubated with UDPglucuronate and a Triton X-100-treated rat liver microsomal preparation. If by the glucuronidation of bilirubin monoglucuronide the same preference for glucuronidation of R_1 should exist, the azodipyrrole glucu-

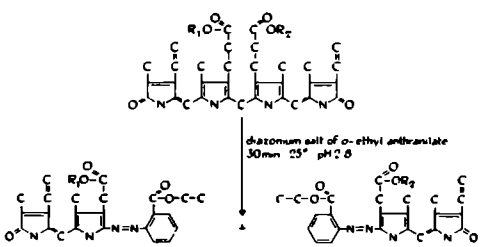

Fig. 6. The formation of azo pigments in the diazo reaction.

TABLE III

The site of attachment of the glucuronyl group by the conversion of bilirubin into monoglucuronide and by the conversion of mono- into diglucuronide. Bilirubin (83 μM) was incubated with UDPglucuronate (3 mM) and a Triton X-100 (0.25%, v/v)-treated microsomal preparation from rat liver for 10 and 20 min at 37°C. Bilirubin monoglucuronide (19.9 and 17.9 μM) was incubated with UDPglucuronate (3 mM) and a solubilized preparation from cat liver for 0 and 14 min at 37°C. After incubation, the diazo reaction was performed and the azo pigments were separated by thin-layer chromatography. Subsequently the azodipyrrole-glucuronide pigment was treated with methanol and H_2SO_4 and the vinyl/isovinyl ratio was determined as described by Jansen and Stoll [13]. B, BMG and BDG represent bilirubin, mono- and diglucuronide. BMG and BDG are expressed as nmoles/ml incubation medium.

Substrate	Enzyme preparation	pH	Time	BMG	BDG	Azodipyrrole—glucuronide vinyl/isovinyl ratio
B	Wistar rat	8.1	10	9.6	0	3.8
			20	18.5	0	3.3
BMG	Cat	8.1	0	19.9	0.6	3.1
			14	14.2	9.8	1.8
BMG	Cat	6.5	0	17.9	0.5	3.7
			14	10.7	7.7	2.1

ronide vinyl/isovinyl ratio should increase as a result of the second step reaction. In Table III it is seen that this was not the case.

Formation of bilirubin diglucuronide in vivo

In our previous communication it has been reported that the bilirubin moiety of diglucuronide, formed by means of isomerisation of monoglucuronide, has to a significant extent the isomeric structure of bilirubin XIIIα [8]. In order to study which mechanism accounts for the formation of diglucuronide in vivo, the isomeric structure of the alkaline-labile bilirubin conjugates in cat and rat bile was analyzed. Azo pigment analysis revealed that 70 - 100% of the bilirubin derivatives in cat bile and 38 - 50% of the bilirubin derivatives in rat bile were diglucuronides. From Fig. 7 it can be derived that the alkaline-labile bilirubin conjugates in bile of cats and rats have predominantly the IXα isomeric structure, suggesting that both in cats and rats, diglucuronide is formed enzymically.

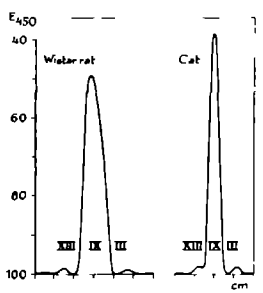

Fig. 7. Bilirubin isomers in bile from Wistar rat and cat. After hydrolysis of the bilirubin glucuronides the bilirubin isomers were analyzed according to the method of McDonagh and Assisi [12]. The picture represents the scan of the silica gel plate The remittance at 450 nm is depicted on the ordinate and the migration distance (from left to right) on the abscissa.

Discussion

In vitro, bilirubin diglucuronide can be formed in two ways In the presence of a solubilized preparation from cat liver microsomes, however, monoglucuronide was only converted into diglucuronide after addition of UDPglucuronate. Diglucuronide formation by means of isomerisation of monoglucuronide was inhibited in the presence of a certain amount of enzyme protein. The stoichiometry of the isomerisation and the UDPglucuronate-dependent reaction were considerably different, as in the latter instance 1 mole of monoglucuronide was converted into 1 mole of diglucuronide It was furthermore found that the tetrapyrrole structure of these compounds stayed the same in this process, in contrast to the changes observed by the isomerisation reaction. These results evidently show that, besides diglucuronide formation by means of isomerisation, diglucuronide may be formed by an enzyme-catalyzed transfer of the glucuronyl group from UDPglucuronate to the monoglucuronide. Analysis of cat and rat bile suggests that diglucuronide formation by means of the isomerisation reaction is not an important mechanism in the liver of these animals.

The present study substantiates our previous findings that the formation of bilirubin diglucuronide occurs in two distinct steps [6]. Indications were found that these steps are catalyzed at different active sites. At pH 8.1 unconjugated bilirubin was converted into bilirubin diglucuronide and it was shown that at this pH both glucuronidation steps can be mediated. Another pH optimum for the conversion of mono- into diglucuronide was found at pH 6.5, and at this pH unconjugated bilirubin was hardly accepted as substrate. These findings suggest the existence of a bilirubin glucuronyltransferase with an optimum at pH 8 1 and a bilirubin monoglucuronide glucuronyltransferase with an optimum at pH 6 5.

The question whether the conversion of mono- into diglucuronide at pH 8.1 takes place at the same active site as the conversion of bilirubin into its monoglucuronide, is difficult to answer It was found that *p*-nitrophenol inhibits the conversion of mono- into diglucuronide at pH 8 1 in a competitive way whereas the first glucuronidation step was not influenced by this aglycone [6] This is an indication that besides a bilirubin monoglucuronide glucuronyltransferase with an optimum at pH 6.5 there may be another active site for this reaction with an optimum at pH 8.1, different from the above mentioned bilirubin glucuronyltransferase. This assumption is substantiated by our finding that with a microsomal preparation from Wistar rat liver or with the solubilized fraction from it, only the first bilirubin glucuronidation step is mediated at pH 8 1 (unpublished results). This suggests that the site for the mediation of the second step at high pH, is deficient in Wistar rat liver. Use was made of this fact with the preparation of bilirubin monoglucuronide in the present study. Halac et al. [5] found that upon incubation of unconjugated bilirubin with a rat liver microsomal preparation at pH 8.0, the diglucuronide was formed in excess over the monoglucuronide. This suggests that their Fischer strain rats differ in this respect from Wistar rats (also used in ref. 4) and from Sprague—Dawley rats [17,18].

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References

1 Fevery, J., Van Hees, G.P., Leroy, P., Compernolle, F. and Heirwegh, K.P.M. (1971) Biochem. J. 125, 803 - 810
2 Fevery, J., Van Damme, B., Michiels, R., De Groote, J. and Heirwegh, K.P.M. (1972) J. Clin. Invest. 51, 2482 - 2492
3 Kuenzle, C.C. (1970) Biochem. J. 119, 411 - 435
4 Fevery, J., Leroy, P., Van de Vijver, M. and Heirwegh, K P.M. (1972) Biochem. J. 129, 635 - 644
5 Halac, E., Dipiazza, M. and Detwiler, P. (1972) Biochim Biophys. Acta 279, 544 - 553
6 Jansen, P.L.M. (1972) Folia Med. Neerl 15, 207 - 213
7 Dutton, G.J. (1971) Handbook of Experimental Pharmacology Vol. XXVIII, Part 2, Concepts in Biochemical Pharmacology, pp. 378 - 400, Springler Verlag, Berlin
8 Jansen, P.L.M. (1973) Clin. Chim. Acta, in the press
9 Halac, E and Reff, A (1967) Biochim. Biophys. Acta 139, 328 - 343
10 Van Roy, F P. and Heirwegh, K P.M. (1968) Biochem. J. 107, 507 - 518
11 Ostrow, J.D. and Murphy, N.H. (1970) Biochem. J. 120, 311 - 327
12 McDonagh, A.F. and Assisi, F (1971) FEBS Lett. 18, 315 - 317
13 Jansen, F.H. and Stoll, M S (1971) Biochem J. 125, 585 - 597
14 Lowry, O H., Rosebrough N J., Farr, A. L and Randall, R.J (1951) J. Biol. Chem. 193, 265 - 275
15 Vessey, A. and Zakim, D (1972) J. Biol. Chem. 247, 3023 - 3028
16 Jansen, F.H and Billing, B.H (1971) Biochem. J. 125, 917 - 919
17 Black, M., Billing, B H. and Heirwegh, K.P.M (1970) Clin Chim. Acta 29, 27 - 35
18 Strebel, L. and Odell, G.B. (1969) Pediatr. Res. 3, 351 - 352

DELIPIDATION AND REACTIVATION OF *UDP*GLUCURONYLTRANSFERASE FROM RAT LIVER

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UDPglucuronyltransferase was solubilized by treating Wistar rat liver microsomes with deoxycholate. Chromatography of this preparation on Bio-Gel P 30 resulted in extraction of 92% of phospholipids and complete loss of enzyme activity. UDPglucuronyltransferase was reactivated by dialysing this delipidated preparation in the presence of lecithin, a mixture of liver microsomal lipids or liver microsomal membranes from homozygous UDPglucuronyltransferase deficient Gunn rats. Phosphatidylserine or phosphatidylethanolamine were without effect.

Virtually complete enzyme reactivation was obtained with regard to glucuronidation and glucosidation of bilirubin; however, the inactivation of UDPglucuronyltransferase with p-nitrophenol as substrate was irreversible.

These findings demonstrate that UDPglucuronyltransferase with bilirubin as substrate is a lipid-requiring enzyme.

UDPglucuronyltransferase (UDPglucuronate glucuronyltransferase, EC 2.4.1.17) catalyzes the glucuronyl conjugation of several small molecular compounds. The enzyme is important in conjugation of hormones, products of endogenous metabolism, drugs and toxic compounds[1]. Many investigators have tried to determine whether glucuronidation of these compounds is mediated by a single enzyme or whether different UDPglucuronyltransferases exist[2,3,4,5,6]. Attemps to separate and purify different UDPglucuronyltransferases have been unsuccessful[7,8,9].

UDPglucuronyltransferase is associated with hepatic endoplasmic reticulum membranes. Enzyme solubilization has been tried with ultrasonication and treatment with digitonin, various snake venoms, deoxycholate and lubrol[7,8,9,10,11]. The 100,000 x g supernatant fraction of microsomes treated with ultrasonication or deoxycholate contains enzymatically active membrane fragments. Preparations not containing these fragments are inactive[7]. With these preparations as starting material, UDPglucuronyltransferase was purified to a limited extent[7,8,9].

Treatment of microsomes with phospholipase A inactivates UDPglucuronyltransferase and activity is restored upon addition of phospholipid micelles[12,13] indicating that lipid is probably required for UDPglucuronyltransferase activity. Purification of membrane proteins inevitably results in dissolution of their lipid environment. During purification of cytochrome P-450[14,15] cytochrome b_5[16] and NADH cytochrome b_5 reductase[17] gel filtration and ion exchange techniques in the presence of detergents disturb protein-lipid relationships; the purified enzymes were in delipidated, non-particulate fractions and enzyme activity was reduced[14,15,16,17].

These enzymes function normally when bound to microsomal membranes or in the presence of phospholipid[14,15,18,19].

In the present study, UDPglucuronyltransferase activity was fully restored on addition of lipid fractions to a delipidated, inactive preparation. An understanding of this problem is considered essential for ultimate purification and functional characterization of the enzyme protein.

Animals

Adult male Wistar and homozygous UDPglucuronyltransferase deficient (Gunn) rats, weighing 200 - 250 g, were used, and were fed rat breeder chow and water ad libitum.

Chemicals

Bilirubin, uridine 5' diphosphoglucuronic acid (ammonium salt), and uridine 5' diphosphoglucose were obtained from Sigma, St. Louis, Mo; p-nitrophenol was from Calbiochem, San Diego, Calif; glycerol was from Fisher Scientific Company, Fair Lawn, N.J; deoxycholic acid, sodium salt was obtained from Sigma, St. Louis, Mo.; and ethyl anthranilate was obtained from Eastman Kodak Company, Rochester, N.Y. For phosphorus quantitation, the Fiske and Subarrow reducer from Sigma, St. Louis, Mo., was used. Trizma Base, Sigma, St. Louis, Mo. was used for preparation of buffers. Glacial acetic acid, analytical grade, was from Mallinckrodt Chemical Works, St. Louis, Mo. All buffers were prepared with deionized distilled water. Bovine serum albumin was obtained from Sigma, St. Louis, Mo. Egg L-α-Lecithin, chromatographically pure, was obtained from General Biochemicals, San Diego, Calif. Lysofree A grade, synthetic L-α-cephalin (β, γ dipalmitoyl) and purified bovine brain phosphatidyl serine, A grade, were obtained from Calbiochem, San Diego, Calif.

Bio Gel P30 (100-200 mesh) was obtained from Bio Rad, Richmond, Calif. For thinlayer chromatography, glass plates precoated with Silica Gel G, layer thickness 0.25 mm, size 20 x 20 cm (Analtech Inc.) distributed by Fisher Scientific Company, Pittsburgh, Pa. were used.

Preparation of microsomes

All procedures were performed in a cold room at 4°C. Wistar rats and homozygous Gunn rats were killed by decapitation under light ether anesthesia. The livers were removed immediately, perfused with 50 ml ice-cold saline and cut into small pieces. A 15% (w/v) homogenate was prepared with a Teflon-glass homogenizer in 0.2 M Tris acetate buffer, pH 8.1, containing 0.25 M sucrose and 0.2 mM disodium EDTA. The homogenate was centrifuged at 650 x g for 15 min. in swinging buckets (IEC PR-J centrifuge, Damon IEC Division). The supernatant was centrifuged twice at 9000 x g for 15 min (rotor, SS-34; Servall, superspeed, automatic refrigerated centrifuge, RC-2, Sorvall, Newton, Conn.). The fatty layer on top of the 9000 x g supernatant was carefully removed by suction and a microsomal fraction was prepared from the remaining supernatant by centrifugation at 100,000 x g for 60 min (Beckmann L2-65 B ultracentrifuge) in pre-weighed tubes. After centrifugation, the supernatant was removed, the tubes were reweighed and the pellets were resuspended with a few strokes of the Teflon-glass homogenizer in 4 ml 0.02 M Tris acetate buffer, pH 8.1: glycerol (9:1 v/v) per gram of pellet. The suspensions were stored in a liquid nitrogen freezer (-180°C). The microsomal fraction from Wistar and Gunn rat liver were stored up to three weeks. Storage did not alter UDPglucuronyltransferase activity with bilirubin or p-nitrophenol as substrates.

Solubilization and delipidation

Microsomal suspensions from Wistar rat liver were thawed at 4°C and 1 ml 0.02 M Tris acetate buffer, pH 8.1: glycerol (9:1, v/v) containing 15 mg deoxycholate was added per 4 ml suspension. After standing for 20 min at 4°C, this suspension was centrifuged at 100,000 x g for 60 min. Centrifugation resulted in a clear supernatant, a loose cloudy fraction at the

bottom of the tube and a small translucent pellet. Only the clear supernatant was processed further. Bovine serum albumin was added to the supernatant fraction (4 mg/ml). Two samples of 4 ml each were applied to two identical columns (Pharmacia K26/70, 2.6 x 70 cm, bed volume 370 ml) previously packed with Bio Gel P30 which was equilibrated with 0.02 M Tris acetate, pH 8.1: glycerol (9:1, v/v) containing 4.8 mM sodium deoxycholate, 0.1 M NaCl and 0.1 mM disodium EDTA (modification of the procedure by Rogers and Strittmatter[19]). The same buffer was used for elution.

Enzyme Reactivation

Immediately after elution from Bio Gel, samples with the highest protein concentration were combined with a mixed lipid preparation, commercial phospholipids or Gunn rat liver microsomes. The protein-lipid or the protein-microsome mixtures were dialyzed against 4 Liters of 0.02 M Tris acetate pH 7.25: glycerol (97.4: 2.6, v/v) containing 5 mM $MgCl_2$. Dialysis was performed for 6 hours at 18°C and for 16 hours at 4°C.
After dialysis, the fractions were diluted (1:1, v/v) with 0.02 M Tris acetate, pH 8.1 containing 0.25 M sucrose and 0.2 mM disodium EDTA. These solutions were centrifuged at 100,000 x g for 60 min. The resulting pellet was resuspended in Tris-sucrose-EDTA buffer with a few strokes of the Teflon-glass homogenizer.

The enzyme assay was immediately performed.

Lipid preparations

Lipids were extracted from microsomal preparations from Wistar rat liver by the method of Folch et al[20]. The organic solvent was evaporated from the lipid extract by a stream of nitrogen while keeping the solution at 37°C. The lipids were dispersed in 0.02 M Tris acetate, pH 8.1: glycerol (9:1, v/v)

by ultrasonication (Sonifier Cell Disruptor, Model W 140, Heat Systems and Ultrasonics, Inc., Plainview, N.Y.). Phospholipids from commercial preparations were dissolved in the same way.

Enzyme assay

The conjugation of bilirubin with glucuronic acid was determined exactly as decribed previously[21]. Incubation mixtures (volume: 1.2 ml) contained 0.2 ml enzyme preparation and 83 µM bilirubin, 3mM UDPglucuronate and 3.3 mM $MgCl_2$. For determination of bilirubin glucosidation, UDPglucuronate was replaced by 3 mM UDPglucose. The mixtures were incubated at 37°C in a shaking water bath for 20 min. Diazo reaction and measurement of the azopigments were performed as described by Fevery et al[22]. Nature of the azopigments was confirmed by thin-layer chromatography[22].
The assay for p-nitrophenol glucuronidation was according to Henderson and Kersten[23]. Incubation mixtures (volume: 0.6 ml) contained 0.1 ml enzyme preparation and 1.66 mM p-nitrophenol, 3mM UDPglucuronate and 3.3 mM $MgCl_2$. The mixtures were incubated for 20 min. at 37°C.

Other determinations

Protein was determined according to Lowry et al[24]. The phosphorus assay of Bartlett et al.[25] was used. Multiplication of the phosphorus concentration by the conversion factor 25 gave the phospholipid concentration.
Phospholipid analysis was performed by thin-layer chromatography with chloroform:methanol:water (65:25:4 v/v/v) as solvent system. After development, the plates were sprayed with ninhydrin (0.25%, w/v) in 90% butanol, heated for 5 min. at 90°C, sprayed again with 80% sulfuric acid, and heated for 10 min at 120°C prior to examination.

As shown in Table I, total activity (activity in pellet + supernatant) with regard to glucuronidation of p-nitrophenol and bilirubin, increased considerably as a result of deoxycholate treatment. Maximal activation occured at 10 mg deoxycholate per g microsomal pellet. No activation was found for glucosidation of bilirubin. For maximal solubilization, a somewhat higher amount of deoxycholate, 15 mg per gram microsomal pellet, was required. Higher deoxycholate concentrations were inhibitory for glucuronidation of p-nitrophenol and bilirubin. Supernatant glucosidation was not significantly different at 10, 15 and 20 mg deoxycholate per gram microsomal pellet.

The data in Table I are ratios calculated on basis of activity per ml enzyme suspension. Calculation of ratios on basis of activity per mg microsomal protein, yielded similar results. Deoxycholate treatment did not result in selective solubilization of UDPglucuronyltransferase nor of the glucosyl transferase activity. These enzymes were simply solubilized with the bulk of the microsomal proteins. Bovine serum albumin was added to the solubilized fraction (microsomal protein : albumin was approximately 2:1, w/w) and this solution was applied to a Bio Gel P30 column. Elution with a deoxycholate containing buffer resulted in separation of the microsomal proteins and phospholipids (Figure 1). The elution buffer contained 10% glycerol. At 20% glycerol, no separation was obtained. In the absence of glycerol or albumin, enzyme, activity was irreversibly destroyed. Chromatography on Bio Gel P30 resulted in extraction of 92% of the phospholipids. Thin layer chromatography of the Folch extract from the delipidated preparation revealed only one clear circumscribed spot with the same Rf and color as glycerol.

The delipidated protein was completely inactive enzymatically (Table II). Dialysis of delipidated protein together with mixed lipid extracted from Wistar rat microsomes, reactivated bilirubin glucuronidation. The reactivated preparation was concentrated by centrifugation and precipitated by centrifugation at 100,000 x g (Table II).

Dialysis of the delipidated protein together with microsomes from UDPglucuronyltransferase deficient Gunn rat liver also resulted in an enzymatically active preparation (Table III). Reactivation only occured when delipidated protein and lipid preparations or Gunn rat liver microsomes were dialyzed together. Simple addition of the lipid preparation or Gunn rat microsomes to non-dialyzed delipidated protein or to the protein after dialysis did not result in reactivation. To rule out reactivation of Gunn rat microsomal protein, Gunn rat liver microsomes were mixed with protein-free deoxycholate-containing elution buffer (see Material and Methods). This suspension was dialyzed against 0.02 M Tris acetate, pH 7.25: glycerol (97.4: 2.6, v/v) containing 5 mM $MgCl_2$ which did not result in reactivation.

The optimal phospholipid / delipidated protein ratio was determined by adding varying amounts of mixed lipid or Gunn rat microsomes to a constant amount of delipidated protein (Figure 2). The optimal ratio was approximately 1 when mixed lipids were used for reactivation, and approximately 2 when Gunn rat liver microsomes were used.

Commercial preparations of lecithin, phosphatidyl serine and phosphatidyl ethanolamine were tested as reactivators of the delipidated preparation (Table IV). No reactivation was found with phosphatidyl ethanolamine. Lecithin was the most potent reactivating phospholipid preparation. Slight activity was found with phosphatidyl serine. However, thin layer chromatography revealed that the phosphatidyl serine preparation contained a small amount of lecithin. The phosphatidyl ethanolamine preparation was contaminated with a small amount of

phosphatidyl serine. The lecithin preparation was chromatographically pure. A lecithin/protein ratio of about 1 appeared to be optimal for both glucuronidation and glucosidation of bilirubin (Figure 3).

Marked differences were found when enzyme activity in the reactivated preparations was measured with different substrates. The preparation was completely inactive with p-nitrophenol as substrate for glucuronidation, (Table V). Relatively high activity was found for glucosyl conjugation of bilirubin. The activity of the lecithin reactivated preparation amounted to 181% as compared with bilirubin glucosidation activity in normal microsomes. Reactivation with respect to glucuronidation of bilirubin amounted to 90% when the optimal lecithin/protein ratio was used.

The stability of the enzyme protein in the absence and presence of lecithin was tested. Delipidated protein was incubated at 37^{o} for 24 and 48 min , subsequently mixed with lipid and dialyzed. The activity of this preparation was compared with a preparation incubated under the same circumstances after reactivation. The former preparation was considerably more labile than the latter (Table VI). Upon reactivation, the reaction rate remained constant for at least 20 min (Figure 4).

TABLE I *ACTIVATION AND SOLUBIZATION OF UDPGLUCURONYLTRANSFERASE*

Substrates	%Deoxycholate (w/w)	total activity	activity in supernatant
1. bilirubin + UDPglucuronate	0	1.00	0
	0.5	3.18	0.12
	1.0	3.36	1.51
	1.5	2.76	1.85
	2.0	1.78	1.26
2. bilirubin +UDPglucose	0	1.00	0
	0.5	0.78	0
	1.0	1.03	0.31
	1.5	0.37	0.20
	2.0	0.33	0.33
3. p-nitrophenol +UDPglucuronate	0	1.00	0
	0.5	6.55	1.63
	1.0	9.63	3.73
	1.5	7.50	4.88
	2.0	5.66	2.81

Samples (2ml) from a microsomal suspension from Wistar rat liver (0.25 g microsomes / ml) were mixed with 0.5 ml of a deoxycholate solution of varying concentrations. Samples and deoxycholate contained 0.02 M Tris acetate buffer pH, 8.1: glycerol (9:1, v/v). After standing for 20 min at 4°, the samples were centrifuged at 100,000 x g for 60 min. Enzyme activities were measured in the clear supernatant and in the pellet. The pellet was resuspended in a final volume of 2.5 ml. Total activity represents activity in pellet + supernatant. Activities were calculated per ml enzyme suspension. The activity of the resuspended untreated pellet was called 1.00.

TABLE II *REACTIVATION OF BILIRUBIN GLUCURONIDATION WITH MIXED MICROSOMAL LIPID*

	nmoles/mg protein	mg protein/ml	phospholipid/protein
delipidated protein	0	3.78	0.06
reactivated preparation			
-after dialysis	2.98	3.01	0.77
-after centrifugation			
supernatant	0	1.81	0.33
pellet	4.00	4.55	1.14

Delipidated microsomal protein was dialyzed together with mixed microsomal lipids as described in Material and Methods. After dialysis, an aliquot was removed for enzyme assay. The remainder was diluted 1:1 and centrifuged at 100,000 x g for 60 min. Enzyme activity was measured in supernatant and resuspended pellet.

TABLE III *REACTIVATION OF BILIRUBIN GLUCURONIDATION BY MICROSOMAL MEMBRANES FROM THE GUNN RAT LIVER*

	nmoles/mg protein	mg protein/ml	phospholipid/protein
delipidated protein	0	3.78	0.06
Gunn rat microsomes	0	15.40	0.59
reactivated preparation			
-after dialysis	1.04 (2.49)	8.80	0.51
-after centrifugation			
supernatant	0	4.05	0.18
pellet	0.85	20.00	0.44

Delipidated microsomal protein from a Wistar rat was dialyzed with a microsomal preparation from Gunn rat liver and processed as described in the legend of Table II. The value between brackets represents enzyme activity per mg Wistar rat protein. A correction was made for the presence of an estimated amount of Gunn rat liver microsomal protein.

TABLE IV *REACTIVATION OF BILIRUBIN GLUCURONIDATION BY DIFFERENT PHOSPHOLIPID PREPARATIONS*

	nmoles/mg protein	phospholipid/protein
mixed lipid	6.15	1.29
lecithin	6.24	2.97
phosphatidyl serine	1.29	2.34
phosphatidyl ethanolamine	0	2.95

Delipidated microsomal protein was dialyzed with different phospholipid preparations. Bilirubin glucuronidation was assayed immediately after dialysis.

TABLE V *CONJUGATION OF BILIRUBIN AND P-NITROPHENOL WITH NORMAL MICROSOMES AND REACTIVATED PREPARATIONS*

	bilirubin +UDPglucuronate	bilirubin +UDPglucose	p-nitrophenol +UDPglucuronate	phospholipid/ protein
microsomes	12.00	1.63	474	0.78
delipidized protein	0	0	0	0.06
reactivated with mixed lipid %reactivation	4.71 39	1.27 78	0	1.19
reactivated with lecithin %reactivation	10.79 90	2.43 181	0	1.02

Glucuronidation of bilirubin and p-nitrophenol and glucosidation of bilirubin were determined in normal microsomes and in reactivated preparations.

TABLE VI *STABILITY OF THE DELIPIDIZED PREPARATION BEFORE AND AFTER REACTIVATION*

Incubation at 37°	-lecithin	+lecithin	ratio
0	8.22	8.22	1
24	4.69	7.15	0.66
48	0.36	4.59	0.08

The delipidated preparation was incubated for 0,24 and 48 min at 37°, subsequently mixed with lecithin and dialyzed. Another sample of the delipidated preparation was mixed with lecithin, dialyzed and subsequently incubated at 37°.

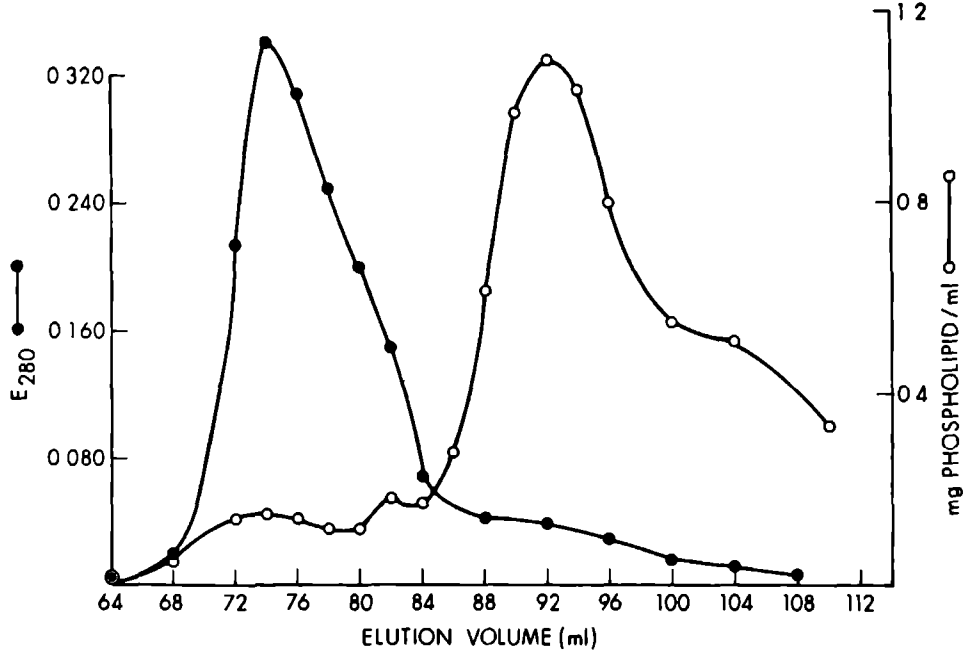

Figure 1. *Separation of microsomal proteins and phospholipids following chromatography on Bio Gel P30. Wistar rat liver microsomes wetreated with deoxycholate (15 mg deoxycholate/gram microsomal pellet) and chromatography of the 100,000 x g supernatant was performed. The chromatography column contained Bio Gel P30. As equilibration and elution buffer, 0.2 M Tris acetate, pH 8.1: glycerol (9:1, v/v) containing 4.8 sodium deoxycholate, 0.1 M NaCl and 0.1 mM disodium EDTA, was used. Before application to the column, the sample was mixed with bovine serum albumin (4 mg/ml).*

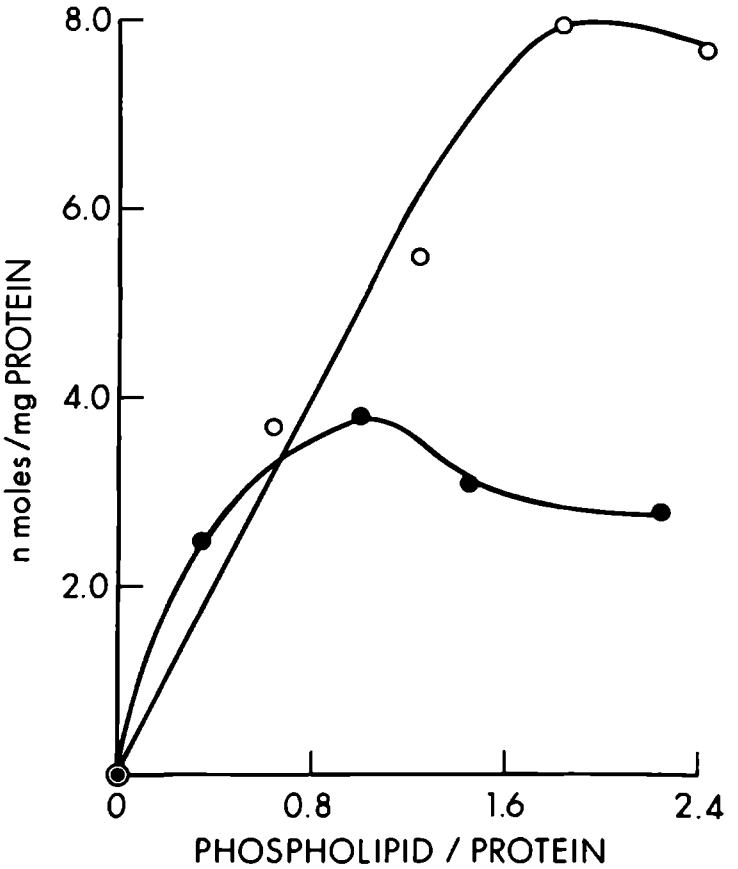

Figure 2. *Bilirubin glucuronidation with preparations reactivated by different amounts of mixed lipid or Gunn rat microsomal membranes. Delipidated Wistar rat microsomal protein was mixed with different amounts of mixed lipid or Gunn rat microsomal membranes and dialyzed. After dialysis the preparations were centrifuged at 100,000 x g for 1 hour and bilirubin glucuronidation was measured in the pellet.*

O —— O reactivated with Gunn rat microsomes

● —— ● reactivated with mixed microsomal lipids

The ordinate represents enzyme activity per mg Wistar rat protein and the abscissa represents the phospholipid/Wistar rat protein ration. The data were corrected for the presence of Gunn liver microsomal protein.

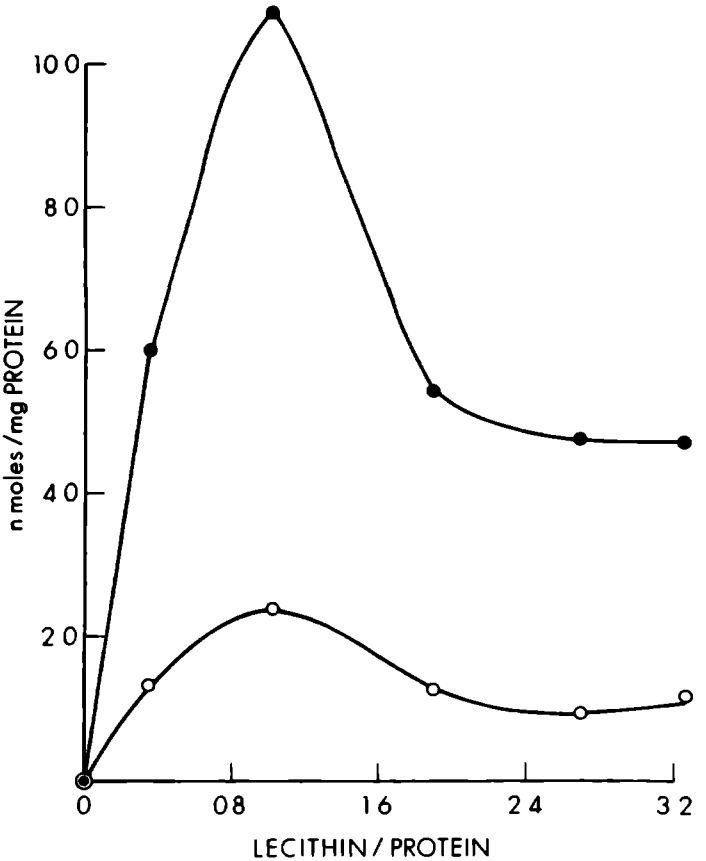

Figure 3. *Glucuronidation and glucosidation of bilirubin by preparations reactivated with different amounts of lecithin. Delipidated microsomal protein was mixed with different amounts of lecithin and dialyzed. After dialysis, the preparations were centrifuged at 100,000 x g for 1 hour. Glucuronidation and glucosidation of bilirubin were measured in the resuspended pellet.*
● —— ● bilirubin glucuronidation;
O —— O bilirubin glucosidation.

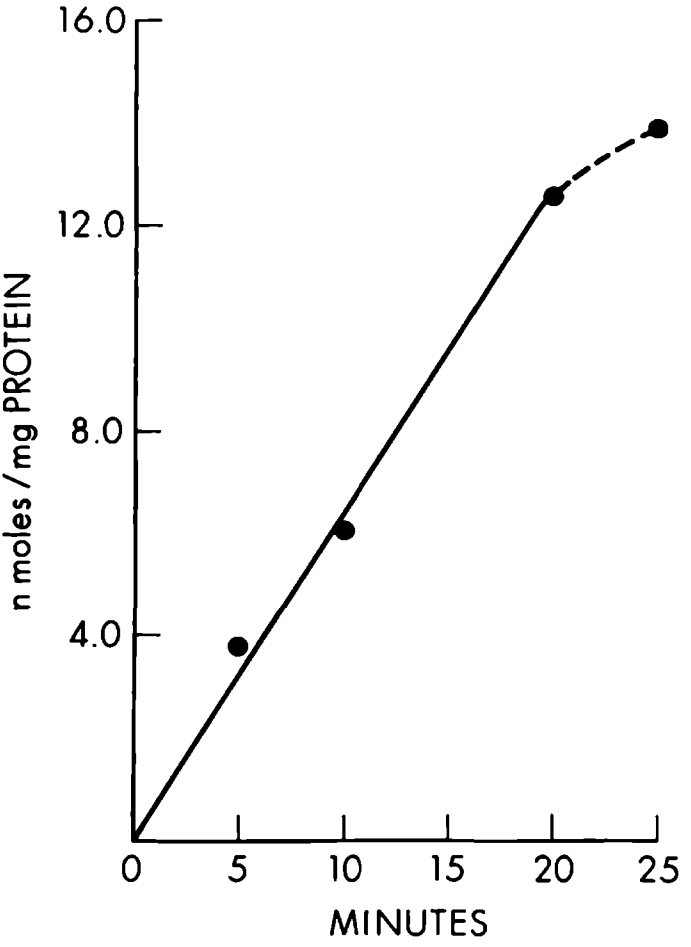

Figure 4. *Bilirubin glucuronidation as function of incubation time. Delipidated protein was mixed with lecithin in the optimal lecithin/protein ratio. After dialysis and centrifugation (100,000 x g, for 1 hour), bilirubin glucuronidation was measured in the resuspended pellet.*

UDPglucuronyltransferase is solubilized in an active form by treating rat liver microsomes with deoxycholate[8,26]. Chromatography on Bio Gel P30 extracted 92% of phospholipids from this preparation. Similar chromatography of digitonin solubilized preparations resulted in less complete protein-lipid separation. On removal of deoxycholate from the delipidated protein by dialysis, extensive precipitates were formed in the dialysis bags, indicating insolubility of the proteins. The reactivated UDPglucuronyltransferase preparation was also insoluble and easily concentrated by centrifugation. Phospholipids are required for the function of many microsomal enzymes. Lecithin is required for reduction of cytochrome P-450[18]; a lecithin-lysolecithin mixture reactivates delipidated NADH-cytochrome reductase[27]; and phosphatidylethanolamine is required for glucose 6-phosphatase activity[28]. In the present study, three phospholipids were tested. Lecithin, but not phosphatidylserine or phosphatidylethanolamine, reactivated UDPglucuronyltransferase activity in a delipidated preparation. Reactivation was obtained only after dialysing the delipidated protein together with phospholipid or Gunn rat liver microsomes. Simple addition of phospholipid or Gunn rat microsomes to delipidated protein did not result in reactivation. This suggests that the enzyme protein must bind to phospholipid or microsomal membranes in order to regain UDPglucuronyltransferase activity. The lecithin-bound form was more stable at 37° than the free form. The function of lecithin may be to confer specific conformation to the enzyme protein which renders it enzymatically active and stable[29].

The substrate specificity of preparations reactivated with mixed phospholipids or lecithin was tested. These preparations catalyzed the formation of bilirubinglucuronide but

not p-nitrophenylglucuronide. Glucosidation of bilirubin was also catalyzed by the reactivated preparation. Delipidation irreversibly inactivates UDPglucuronyltransferase activity for p-nitrophenol glucuronidation. This suggests that substrate specificity of UDPglucuronyltransferase is not a function of a phospholipid subunit of the enzyme but that formation of bilirubinglucuronide requires an enzyme protein which does not accept p-nitrophenol as substrate.

1. G.J. DUTTON, (1966) in Glucuronic acid, free and combined (G.J. Dutton, ed.) Academic Press, New York.
2. D. ZAKIM, J. GOLDENBERG and D.A. VESSEY (1973) Biochim. Biophys. Acta 309, 67-74.
3. D.A. VESSEY, J. GOLDENBERG and D. ZAKIM (1973) Biochim. Biophys. Acta 309, 75-82.
4. G.J. MULDER (1971) Biochem. J. 125, 9-15.
5. G.J. MULDER (1972) Biochim. Biophys. Acta 289, 284-292.
6. P.L.M. JANSEN (1974) Biochim. Biophys. Acta 338, 170-182.
7. A.P. MOWAT and I.M. ARIAS (1970) Biochim. Biophys. Acta 212, 65-78.
8. E. HALAC and A. REFF (1967) Biochim. Biophys. Acta 139, 328-343.
9. K.J. ISSELBACHER, M.F. CHRABAS and R.C. QUINN (1962) J. Biol. Chem. 237, 3033-3036.
10. R.D. HOWLAND, A. BURKHALTER, A.J. TREVOR, S. HEGEMAN and D.Y. SCHIRACHI (1971) Biochem. J. 125, 991-997.
11. D.H. GREGORY and R.D. STRICKLAND (1973) Biochim. Biophys. Acta 327, 36-45.
12. A.B. GRAHAM and G.C. WOOD (1969) Biochem. Biophys Res. Commun. 37, 567-575.
13. D. ATTWOOD, A.B. GRAHAM and G.C. WOOD (1971) Biochem. J. 123, 875-882.
14. A.Y.H. LU and M.J. COON (1968) J. Biol. Chem. 243, 1331-1332.
15. W. LEVIN, D. RYAN, S. WEST and A.Y.H. LU (1974) J. Biol. Chem. 249, 1747-1754.
16. A. ITO and R. SATO (1968) J. Biol. Chem. 243, 4922-4930.
17. L. SPATZ and P. STRITTMATTER (1973) J. Biol. Chem. 248, 793-799.

18. H.W. STROBEL, A.Y.H. LU, J. HEIDEMA and M.J. COON (1970) J. Biol. Chem. 245, 4851-4854.
19. M.J. ROGERS and P. STRITTMATTER (1973) J. Biol. Chem. 248, 800-806.
20. J. FOLCH, M. LEES and G.A. SLOANE-STANLEY (1957) J. Biol. Chem. 226, 497-509.
21. P.L.M. JANSEN (1974) Biochim. Biophys Acta 338, 170-182.
22. J. FEVERY, G.P. VAN HEES, P. LEROY, F. COMPERNOLLE and K.P.M. HEIRWEGH (1971) Biochem. J. 125, 803-810.
23. P.Th. HENDERSON and K.J. KERSTEN (1970) Biochem. Pharmacol. 19, 2343-2351.
24. O.H. LOWRY, N.J. ROSENBROUGH, A.L. FARR and R.J. RANDALL (1951) J. Biol. Chem. 193, 265-275.
25. G.R. BARTLETT (1959) J. Biol. Chem. 234, 466-468.
26. B.P.F. ADLARD and G.H. LATHE (1970) Biochem. J. 119, 437-445.
27. P.D. JONES and S.L. WAKIL (1967) J. Biol. Chem. 242, 5267-5273.
28. S.M. DUTTERA, W.L. BYRNE and M.C. GANOZA (1968) J. Biol. Chem. 243, 2216-2228.
29. R. COLEMAN (1973) Biochim. Biophys. Acta 300, 1-3.

The conjugation of bilirubin and p-nitrophenol with glucuronic acid was studied. The question whether UDPglucuronyltransferase is one enzyme which catalyzes the glucuronidation of various compounds, or a group of enzymes in which each enzyme specifically catalyzes certain reactions was the main issue of the study.

First the glucuronidation of various substrates was studied, before and after treatment of test animals with phenobarbital. The glucuronidation activity was tested with microsomal preparations prepared from liver homogenates from Wistar rats, homo- and heterozygous Gunn rats and cats. It was found that the bilirubin glucuronidation of Wistar rat and cat was increased after a twelve day treatment with phenobarbital. The glucuronidation activity determined with p-nitrophenol as substrate was already increased after 6 days phenobarbital. Another difference between the glucuronidation of these two substrates was found with the homozygous Gunn rat. This animal is a mutant unable to form bilirubin glucuronides. Pre-treatment with phenobarbital did not affect their deficient bilirubin glucuronidation capacity. The unimpaired capacity to form p-nitrophenylglucuronide of the homozygous Gunn rat however was largely increased after pretreatment with phenobarbital. These results suggest that different enzymes may play a role in the glucuronidation of the endogenous substrate bilirubin and the artifical substrate p-nitrophenol.

In the literature the assumption prevailed that the glucuronidation of bilirubin is a simple one step reaction and the question how the conjugation of bilirubin with two glucuronic acid molecules is effected has not been answered. The question however is of fundamental importance to understand

the biochemistry of the bilirubin glucuronidation and the interaction of drugs and other foreign compounds with this process.

It was found that incubation of bilirubin and UDPglucuronate with liver microsomes of the cat at pH 8, resulted in the formation of bilirubin mono- and diglucuronide. Relatively more diglucuronide was formed with microsomes treated with Tween 80 than with microsomes treated with Triton X-100 or untreated microsomes. About the same total amount of bilirubin glucuronide was formed with microsomes treated with Tween 80 and Triton X-100. Besides these detergents also albumin influenced the relative amounts of mono- and diglucuronide. With albumin in the incubation medium relatively more mono- and diglucuronide was formed compared with incubations performed in the absence of albumin. These data show that the formation of bilirubin diglucuronide occurs in two steps whereby first the monoglucuronide is formed which is subsequently converted into diglucuronide. These data also show that both steps can proceed at pH 8. With bilirubin monoglucuronide as substrate a bimodal pH curve was found for diglucuronide formation. Equal amounts of diglucuronide were formed at pH 8.0 and pH 6.5.
Incubation of bilirubin and UDPglucuronate with microsomal preparations from Wistar rat liver by pH 8 resulted in the formation of bilirubin monoglucuronide. This in contrast to the results obtained with cat liver. For this reason Wistar rat liver could be used to prepare the substrate bilirubin monoglucuronide biosynthetically.

Incubation of bilirubin monoglucuronide at pH 6.5 in the absence of an enzyme preparation also led to diglucuronide formation. This was a non-UDPglucuronate dependent reaction and could unequivocally be differentiated from the enzymical reaction.

The bilirubin glucuronidating form of UDPglucuronyltransferase proves to be an enzyme which is only active in the presence of phospholipids. A delipidated preparation prepared from microsomes from Wistar rat liver was inactive by the glucuronidation of bilirubin and p-nitrophenol. However only the glucuronidation of bilirubin could be reactivated with phospholipids. The p-nitrophenol glucuronidation capacity of liver microsomes was apparently irreversible destroyed during delipidation. Also these latter results strongly suggest that different glucuronyltransferases exist.

SAMENVATTING

In deze studie werd de conjugatie van bilirubine en p-nitrophenol met glucuronzuur nader onderzocht. Met name de vraag of het UDPglucuronyltransferase één enzyme is, dat de glucuronidering van vele verschillende verbindingen kataliseert, of een groep van enzymen waarbij elk enzyme bij de katalyse van bepaalde specifieke reakties een rol speelt, stond bij de vraagstelling centraal.

Allereerst werd de glucuronidering van verschillende substraten, voor en na behandeling van de proefdieren met phenobarbital, bestudeerd. De glucuronideringsaktiviteit werd getest met microsomale preparaten, bereid uit leverhomogenaten van Wistar rat, homo- en heterozygote Gunn rat en kat. De bilirubine glucuronideringsaktiviteit van Wistar rat en kat was verhoogd na een 12 daagse behandeling met phenobarbital. De glucuronidering van p-nitrophenol was al na 6 dagen phenobarbital verhoogd. Een ander verschil tussen de glucuronidering van deze twee substraten werd gevonden met de homozygote Gunn rat. Dit proefdier kan geen bilirubine glucuronideren, ook niet na voorbehandeling met phenobarbital. De p-nitrophenol glucuronideringskapaciteit van de homozygote Gunn rat was sterk verhoogd na voorbehandeling met phenobarbital. Deze resultaten wijzer erop, dat mogelijk verschillende enzymen een rol spelen bij de glucuronidering van het endogene substraat bilirubine en de lichaamsvreemde stof p-nitrophenol.

Tot nu toe werd in de literatuur er veelal van uitgegaan dat de glucuronidering van bilirubine een simpele éénstapsreaktie is, en de vraag hoe de koppeling van bilirubine met twee moleculen glucuronzuur tot stand komt was niet duidelijk beantwoord. Deze vraag is echter van fundamenteel belang om de biochemie van de bilirubine glucuronidering en de interactie

van lichaamsvreemde stoffen te begrijpen.

Gevonden werd dat inkubatie van bilirubine met levermicrosomen van de kat, in de aanwezigheid van UDPGA, bij pH 8 resulteerde in de vorming van bilirubine homo- en diglucuronide. Met Tween 80 behandelde microsomen werd relatief veel meer diglucuronide gevormd dan met Triton X-100 behandelde of onbehandelde microsomen. Ook albumine was van invloed op de mono-diglucuronide verhouding. Indien de reaktie werd uitgevoerd in de aanwezigheid van albumine bevatte het inkubatie medium aan het eind van de reaktie veel meer mono- dan diglucuronide vergeleken met een reaktie die plaats vond in de afwezigheid van albumine. Deze gegevens duiden erop, dat de vorming van diglucuronide in twee stappen gebeurd, waarbij eerst het monoglucuronide wordt gevormd, dat in een tweede stap in diglucuronide wordt omgezet. Deze gegevens betekenen tevens dat bij pH 8 beide reaktie stappen kunnen plaats vinden. Met bilirubine monoglucuronide als substraat werd een bimodale pH curve gevonden voor de diglucuronide vorming. Bij pH 6.5 en pH 8 werden gelijke hoeveelheden diglucuronide gevormd. Veel minder diglucuronide werd gevormd bij tussen deze twee waarden gelegen pH's.
Inkubatie van bilirubine met levermicrosomen van de Wistar rat bij de pH 8 leidde tot de vorming van voornamelijk bilirubine monoglucuronide. Bij de bereiding van het bilirubine monoglucuronide werd hiervan gebruik gemaakt.
Inkubatie van bilirubine monoglucuronide bij pH 6.5 in de afwezigheid van een enzyme preparaat leidde ook tot diglucuronide vorming. Deze niet-enzymatische reaktie kon op ondubbelzinnige wijze van een enzymatische reaktie worden onderscheiden.

De bilirubine glucuroniderende vorm van het UDPglucuronyltransferase blijkt een enzyme te zijn dat alleen werkzaam is in de aanwezigheid van phospholipiden. Een gedelipideerd preparaat bereid van levermicrosomen van de Wistar rat was on-

werkzaam bij de glucuronidering van p-nitrophenol en bilirubine.
Alleen de bilirubine glucuronidering kon echter met behulp van phospholipiden worden gereaktiveerd. Deze laatste bevinding is een sterk argument dat bij de glucuronidering van deze twee substraten verschillende enzymen betrokken zijn.

CURRICULUM VITÆ

Peter Jansen werd op 3 maart 1946 geboren te 's-Hertogenbosch. Hij bezocht aldaar het St. Janslyceum waar hij in 1964 het diploma HBS-B haalde. Daarna studeerde hij in Nijmegen Geneeskunde. In 1967 werd hij op voordracht van Prof. Dr. Kreuzer, hoofd van de afdeling Fysiologie te Nijmegen, naar de Verenigde Staten uitgezonden waar hij een Summer Research School bezocht in Saranac Lake, New York. In 1970 behaalde hij het semi-arts diploma.

Het onderzoek, neergelegd in dit proefschrift, werd aangevangen in 1971 in het Farmacologisch Instituut te Nijmegen. Het laatste gedeelte van het onderzoek werd uitgevoerd op de afdeling Gastroenterologie van het Albert Einstein College of Medicine te New York, onder leiding van Prof. Dr. I.M. Arias.

De auteur woont momenteel in New York waar hij aan het Albert Einstein College of Medicine een voortgezette training geniet in klinische geneeskunde en wetenschappelijk onderzoek.

STELLINGEN

1. Inductie van UDPglucuronyltransferase door behandeling van proefdieren met fenobarbital of 3-methylcholanthreen leidt tot significante veranderingen in de substraat specificiteit. Dit wijst erop dat UDPglucuronyltransferase uit meerdere enzymen bestaat.

   K.W. Bock, W. Fröhling, H. Remmer en B. Rexer (1973) Biochim. Biophys. Acta 327, 46-56.
   M.M. Jacobson, W. Levin en A. Conney (1974), in press.
   E. Sanchez en T.R. Tephly (1974) Drug Metabolism and Disposition 2, 247-253.
   Dit proefschrift.

2. De omzetting van bilirubine in bilirubine monoglucuronide en van bilirubine monoglucuronide in bilirubine diglucuronide wordt gekatalyseerd door enzymen die op belangrijke punten van elkaar verschillen.

   Dit proefschrift.

3. De isomerisatie van bilirubine en van bilirubine monoglucuronide in een waterig medium geschiedt volgens het mechanisme van de vrije radicaal reactie. Bilirubine monoglucuronide zou in vivo bij obstructieve leverziekten aanleiding kunnen geven tot vorming van potentieel toxische dipyrrylmetheen radicalen.

   A.F. McDonagh (1974) Ann. N.Y. Acad. Sci., in press.
   Dit proefschrift.

4. Implantatie van een stukje lever van een normale rat in de lever van een homozygote Gunn rat leidt tot normalisering van het serum bilirubine gehalte. De conclusie dat dit zou berusten op inductie van het genetisch deficiente UDPglucuronyltransferase is sensationeel maar onwetenschappelijk.

   A.B. Mukherjee en J. Krasner (1973) Science 182, 68-70.

5. Bij het onderzoek naar de interactie van geneesmiddelen met de bilirubine bindingsplaats op albumine, verdient de peroxidase methode van Brodersen de voorkeur boven andere technieken. Alle geneesmiddelen die de placenta kunnen passeren zouden met deze methode gescreend moeten worden.

   R. Brodersen (1974) J. Clin. Invest. 54, 1353-1364.

6. Fototherapie bij icterus leidt niet alleen tot afbraak van bilirubine in wateroplosbare pigmenten, maar ook tot een verhoogde biliaire uitscheiding van ongeconjugeerd bilirubine.

   J.D. Ostrow (1971) J. Clin. Invest. 50, 707-718.
   H.T. Lund en J. Jacobsen (1972) Acta Paediat. Scand. 61, 693-696.

7. De indeling van de glutathion S-transferase in aryl-, alkyl-, alkeen- en epoxide transferasen is obsoleet. De substraat specificiteit van de recent gezuiverde glutathiontransferase is niet met deze indeling in overeenstemming.

   T.A. Fjellstedt, R.H. Allen, B.K. Duncan en W.B. Jakoby (1973) J. Biol. Chem. 248, 3702-3707.
   W.H. Habig, M.J. Pabst en W.B. Jakoby (1974) J. Biol. Chem. 249, 7130-7139.
   M.J. Pabst, W.H. Habig, en W.B. Jakoby (1974) J. Biol. Chem. 249, 7140-7150.

8. Het belangrijkste glutathiontransferase, glutathion S-transferase B, is identiek aan ligandine. Ligandine speelt een rol bij transport van anionen in lever en nier. Mogelijk is conjugatie met glutathion, naast een efficient detoxificatie mechanisme, ook van belang bij deze transportprocessen.

   W.H. Habig, M.J. Pabst, G. Fleischner, Z. Gatmaitan, I.M. Arias en W.B. Jakoby (1974) Proc. Nat. Acad. Sci. USA 71, 3879-3882.

9. Indien de controlegroep in Kellermann's artikel "Aryl hydrocarbon hydroxylase inducibility and bronchogenic carcinoma" uit rokers had bestaan, dan zou het een waardevolle en interessante studie geweest zijn.

   G. Kellermann, C.R. Shaw en M. Luyten-Kellermann (1973) New Engl. J. Med. 289, 934-937.

10. De hepatotoxiciteit van isoniazide (INH) wordt veroorzaakt door een geacetyleerd metaboliet. p-Aminosalicylzuur (PAS) is een remmer van acetyleringsreacties. Daarom is INH in combinatie met PAS waarschijnlijk minder toxisch dan INH bij gebruik alleen of in combinatie met andere tuberculostatica.

    J.R. Mitchell, W.Z. Potter, J.A. Hinson, W.R. Snodgrass, J.A. Timbrell en J.R. Gillette in Toxic Drug Reactions, Handbook of Experimental Pharmacology (1975), in press.

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