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Assay and Properties of Digitonin-Activated Bilirubin Uridine Diphosphate Glucuronyltransferase from Rat Liver

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1. The bilirubin UDP-glucuronyltransferase assay described by Van Roy & Heirwegh (1968) has been improved. 2. Extraction of final azo-derivatives is rendered more simple and efficient by thorough emulsification and by cooling. 3. Pretreatment of homogenates and cell fractions with digitonin increases the sensitivity of the assays and gives less variable results than those with untreated preparations. The activation procedure is flexible. 4. Blank values (obtained from incubation mixtures from which activating bivalent metal ion and UDP-glucuronic acid were omitted) are low. No endogenous conjugate formation could be detected except with untreated, fresh liver homogenates. Control incubation mixtures containing the latter preparations are preferably kept at 0°C. 5. With activated microsomal preparations, rates of breakdown of UDP-glucuronic acid (as monitored by release of P_i) were low. Little if any increase in enzyme activity was found when UDP-*N*-acetylglucosamine was included in the incubation mixtures. 6. Slight deviation from Michaelis–Menten kinetics with respect to bilirubin observed at low substrate concentrations is probably related to the use of binding protein in the assay mixtures. Michaelis–Menten kinetics were followed with respect to UDP-glucuronic acid. Part of the enzyme in microsomal preparations from rat liver functioned independently of added bivalent metal ions. Mn^{2+} was slightly more, and Ca^{2+} somewhat less, stimulatory than Mg^{2+}. The Mg^{2+}-dependent fraction showed Michaelis–Menten kinetics with respect to the added Mg^{2+}. 7. The enzyme activities found were higher than values reported in the literature for untreated or purified preparations from rat liver. They were above reported values of the maximal biliary excretion rate of bilirubin.

Since the demonstration of UDP-glucuronic acid-dependent conjugation of bilirubin (Arias & London, 1957; Grodsky & Carbone, 1957; Lathe & Walker, 1958; Schmid *et al.*, 1957) several developments of the initial assay systems have demonstrated the analytical difficulties involved (Boerth *et al.*, 1965; Van Roy & Heirwegh, 1968). The acceptor substrate, bilirubin, is water-insoluble at pH values below 8 (Brodersen & Theilgaard, 1969), necessitating work at a relatively high pH (Schmid *et al.*, 1957; Wong, 1971) or the use of binding protein (Grodsky & Carbone, 1957; Lathe & Walker, 1958) or organic solvents (Van Roy & Heirwegh, 1968; Adlard & Lathe, 1970) to solubilize the substrate.

Frequently the conjugated bile pigment synthesized *in vitro* is determined after its conversion into azo-pigment derivatives. Completeness of diazo coupling with minimal reaction of excess of unconjugated bilirubin remaining after enzymic incubation was obtained by using diazotized ethyl anthranilate, at pH 2.7, as the coupling reagent (Van Roy & Heirwegh, 1968). The azopigments formed are extracted into an organic solvent. This procedure minimizes erratic blank values due to turbidity and yields preparations that can be analysed rapidly by t.l.c. (Heirwegh *et al.*, 1970; Fevery *et al.*, 1972*b*).

Variable activation of bilirubin UDP-glucuronyltransferase can occur. This may be due to (*a*) variations in the homogenization procedures (Frei, 1970), (*b*) a variable degree of spontaneous activation (Winsnes, 1969) or (*c*) dialysis in alkaline medium (Halac & Reff, 1967). Incorporation of certain substances into the enzymic preparations may also influence the activity of the enzyme, e.g. UDP-*N*-acetylglucosamine (Winsnes, 1969; Adlard & Lathe, 1970), synthetic detergents (Van Roy & Heirwegh, 1968) or digitonin (Heirwegh & Meuwissen, 1968; Winsnes, 1969; Black *et al.*, 1970).

In the present paper an assay of bilirubin UDP-glucuronyltransferase is presented. The method extends the procedures of Van Roy & Heirwegh (1968) using ethyl anthranilate. Digitonin activation minimizes fluctuations of enzyme activity and increases the sensitivity of the procedure. Activities of rat liver homogenates were greater than any values reported in the literature and were comparable with maximal excretion rates. Kinetic analysis showed that the reaction was a complicated one with respect to bilirubin

and demonstrated the existence of an enzyme fraction that was independent of bivalent ions. A modified version of this enzyme procedure has already been found to be applicable to the analysis of needle-biopsy specimens of human liver (Black *et al.*, 1970).

In the present and subsequent papers the term 'azodipyrrole' will be used instead of the currently employed term 'azobilirubin'. The former expresses the chemical nature of the azopigment (derived from unconjugated bilirubin or from the non-conjugated dipyrrole moiety of mono-conjugated bilirubin) more correctly. The term 'azobilirubin' has often led to confusion.

Preliminary accounts of the work have already appeared (Heirwegh & Meuwissen, 1968; Heirwegh *et al.*, 1971).

Materials and Methods

Chemicals

Chemicals were as specified by Van Roy & Heirwegh (1968), except for the following. Chloroform (stabilized with 0.6% ethanol) and crystalline digitonin were from E. Merck A.-G. (Darmstadt, Germany). Human serum albumin (20% w/v; sterile aqueous solution) was obtained from Biotest-Serum Institut G.m.b.H. (Frankfurt a/M, Germany) and UDP-*N*-acetylglucosamine was from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). Salts were from the sources indicated: $MgCl_2,6H_2O$ (Carlo Erba, Milan, Italy); $BaCl_2,2H_2O$, $CaCl_2$, $CoCl_2,6H_2O$ and $FeSO_4,7H_2O$ (E. Merck); $Cd(CH_3CO_2)_2,2H_2O$, $NiCl_2,6H_2O$, $MnCl_2,4H_2O$, $Pb(CH_3CO_2)_2,3H_2O$ and $Zn(CH_3CO_2)_2,2H_2O$ (AnalaR products, from British Drug Houses Ltd., Poole, Dorset, U.K.).

Methods

Cell fractionation. Adult male Wistar R albino rats (body wt. 250–400g) were fed on RMH-B food (Hope Farms, Woerden, The Netherlands) and had free access to water. They were killed by decapitation under light ether anaesthesia. The livers were quickly excised, placed in ice-cold 0.15M-sucrose containing 1mM-EDTA (disodium salt), pH7.4, and cut into small pieces (3–5mm diam.). After decantation of the supernatant fluid fresh sucrose–EDTA medium was added to prepare homogenates and cell extracts (0.25g wet wt. equivalent of liver/ml of suspension) as described by de Duve *et al.* (1955). After centrifugation of the diluted cell extract (0.1g wet wt. equivalent of liver/ml of suspension) in a no. 40 rotor of a Spinco model L-50 ultracentrifuge at $41000g_{av.}$ at 4°C for 7min, the well-packed sediment, together with the overlaying fluffy material, was separated from the supernatant solution. The latter was centrifuged at $80000g_{av.}$ for 23.5min. The sediment was diluted to the initial volume with the sucrose–EDTA medium, resuspended and centrifuged again as described above. The final washed sediment is called the microsomal preparation. The above-mentioned sucrose–EDTA medium was used to dilute all preparations and to resuspend sedimented cell fractions, except when stated otherwise.

Activation of liver homogenate and cell fractions with digitonin. Mixtures of digitonin suspension and of the appropriate enzyme preparations were kept at 0°C for 30min before preparation of the incubation mixtures. (*a*) 1vol. of homogenate or cell extract (0.25g wet wt. equivalent of liver/ml of suspension) was mixed with 1vol. of digitonin suspension (12mg/ml). (*b*) 1vol. of microsomal suspension (0.2g wet wt. equivalent/ml) was treated with 1vol. of digitonin suspension (5.4mg/ml). Digitonin suspensions were prepared by heating the required amounts of digitonin and sucrose–EDTA medium for a few minutes on a boiling-water bath.

Enzymic incubation of digitonin-activated bilirubin UDP-glucuronyltransferase. Incubation mixtures were prepared at 0°C in round-bottomed glass-stoppered centrifuge tubes (11ml). The mixtures contained 200μl of triethanolamine–HCl buffer (0.5M-HCl adjusted with triethanolamine to pH7.7), 40μl of 125mM-$MgCl_2$, 200μl of bilirubin–albumin mixture (see below), 200μl of digitonin-activated enzyme preparation and 20μl of UDP-glucuronic acid soln. (50mg/ml). The components were added in the sequence given except when stated otherwise.

Incubations were performed at 37°C with shaking (frequency: 100 cycles/min; amplitude: 2.35cm) for 5–20min. In general, duplicate assay mixtures were incubated for different periods of time, e.g. 5 and 10min respectively. Controls were incubated in parallel at 37°C and contained 125mM-disodium EDTA, pH7.7, instead of the $MgCl_2$ solution. The bilirubin–human serum albumin mixtures were prepared as described by Black *et al.* (1970), except for the bilirubin concentration, which was decreased to 0.25mg/ml.

Diazotization, extraction and determination of bile pigments. Incubation mixtures were quickly cooled on crushed ice, treated immediately with 2ml of glycine–HCl buffer (0.4M-HCl adjusted to pH2.7 with glycine) and placed in a water bath at 25°C. After 3–5min 1ml of ethyl anthranilate diazo reagent (prepared as described by Heirwegh *et al.*, 1970) was added and coupling was allowed to proceed at 25°C for 30min. The reaction was terminated by adding 0.5ml of freshly prepared ascorbic acid soln. (100mg/ml). After 5–10min the tubes were placed on crushed ice for a few minutes. The contents of each tube were then shaken vigorously (30 times) with 2ml of pentan-2-

one-*n*-butyl acetate (17:3, v/v) and each tube was again cooled for a few minutes. Finally the contents of each tube were mixed very thoroughly with a whirl-mixer (Mixomat; Boskamp, Geräte Bau, Bonn, Germany). Extraction was shown to be most efficient when the aqueous layer became clear before the organic phase. When this was not the case the tubes were placed in deep-freeze at −10° to −20°C for 5–10min and mixed again with the whirl-mixer. Extraction of the pigment was completed by centrifugation for 5min at $1000g_{av.}$ and the E_{546} values of the organic phases were measured in semi-micro-cuvettes (light-path 2cm), with extraction solvent as the reference. Concentrations in 'bilirubin equiv./litre' were calculated from ϵ_{546} 40.7×10^3 litre·mol^{-1}·cm^{-1}. This value was derived from ϵ_{530} (Van Roy & Heirwegh, 1968) by application of the correction factor, $E_{530}/E_{546}=1.09$, obtained by spectrophotometry of extracts containing pure azodipyrrole.

Ancillary determinations. Bilirubin concentrations in bilirubin–albumin mixtures were determined as described by Van Roy *et al.* (1971). The concentration of aqueous solutions of UDP-glucuronic acid was calculated assuming ϵ_{261} 9.9×10^3 litre·mol^{-1}·cm^{-1} at neutral pH (Bock *et al.*, 1956). Protein was determined by the method of Daughaday *et al.* (1952) with albumin as a standard (crystallized bovine plasma albumin; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.). The reference protein contained 2% of water (determined by pycnometry). Inorganic phosphate was measured by the method of Fiske & Subbarow (1925). Azopigment extracts were analysed by t.l.c. (Heirwegh *et al.*, 1970; Fevery *et al.*, 1972*b*).

Results

Choice of incubation and colour-formation controls

The colour of the azopigment extracts obtained from an incubation mixture is due to the following factors: (*a*) conjugated bilirubin synthesized during incubation; (*b*) diazo-positive material present in the enzyme preparation, e.g. conjugated bilirubin present in cholestatic liver; (*c*) diazo-negative compounds

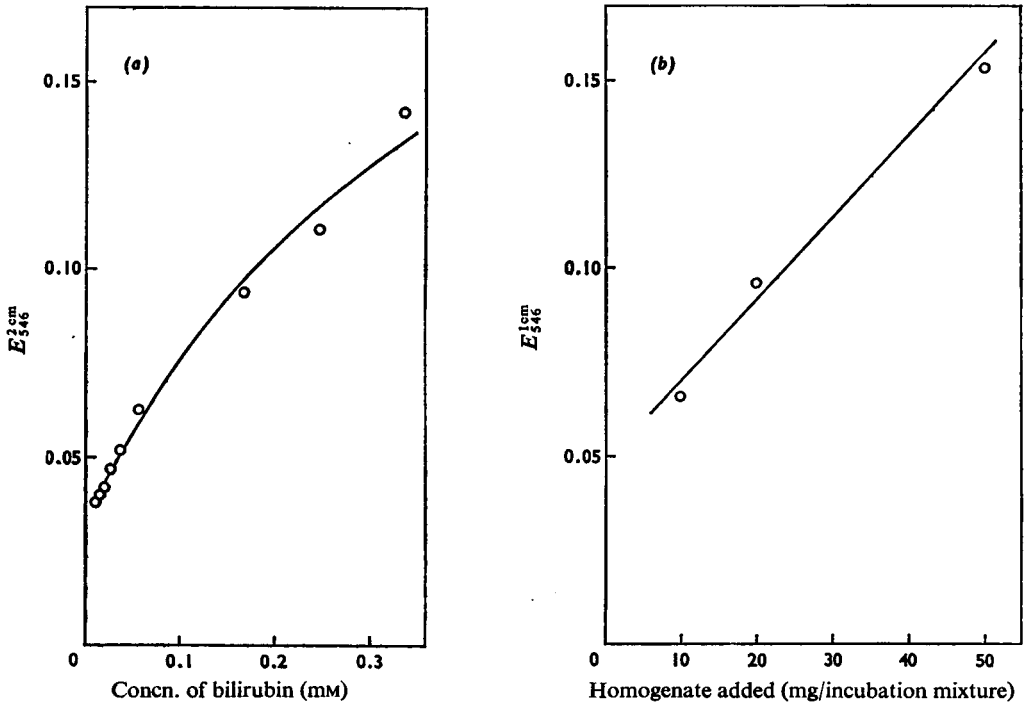

Fig. 1. *Effect of* (*a*) *bilirubin concentration and* (*b*) *the amount of homogenate on blank values*

Control incubation mixtures (UDP-glucuronic acid omitted; Mg^{2+} replaced by disodium EDTA) were assayed as described in the Materials and Methods section. In (*a*) mixtures contained digitonin-activated microsomal material. In (*b*) the concentrations of homogenate and digitonin were varied, their ratio being kept constant at the standard value.

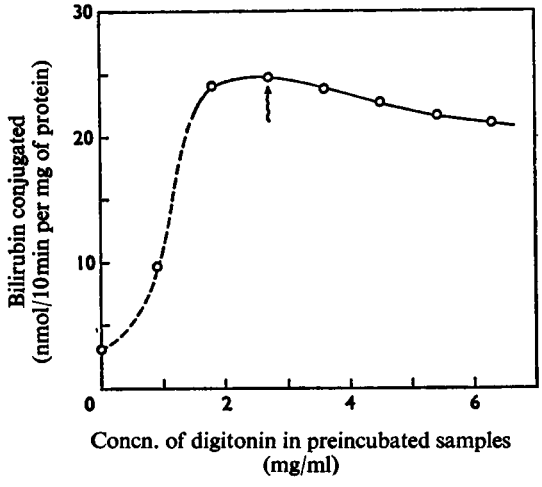

Fig. 2. *Activation of microsomal material at increasing digitonin concentrations*

Mixtures (0.2ml) of a washed microsomal preparation (20mg wet wt. equiv. of liver) and digitonin were kept at 0°C for 30min, followed by enzymic incubation (for 15min at 37°C) and finally by colour formation (see the Materials and Methods section). The arrow indicates the standard digitonin concentration.

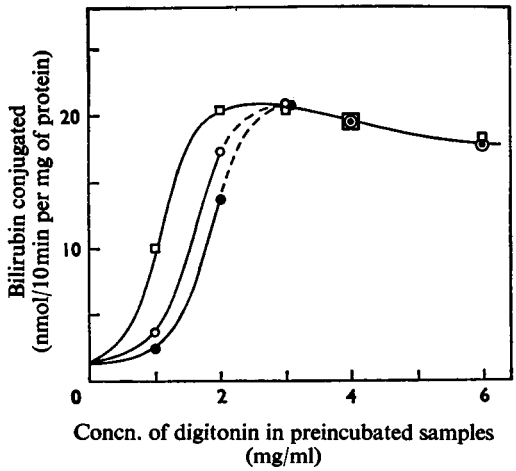

Fig. 3. *Effects of the activation period and of digitonin concentration*

Mixtures (0.2ml) of a washed microsomal preparation (40mg wet wt. equiv. of liver) and digitonin were kept at 0°C for 30min (●), 120min (○) or 19h (□). They were then assayed as indicated in the Materials and Methods section.

absorbing measurably at 546nm; (*d*) turbidity. Controls should be such that (1) no appreciable conjugation occurs during incubation and (2) contributions (*b*), (*c*) and (*d*) are estimated correctly.

Condition (1) was met by omitting the UDP-sugar from the incubation mixtures and by replacing Mg^{2+} by an equivalent amount of disodium EDTA. Demonstration of a Mg^{2+}-independent fraction of UDP-glucuronyltransferase made it necessary to consider whether the measures taken were adequate to suppress eventual conjugate formation from endogenous sugar donors. Control and test incubation mixtures containing fresh unactivated liver homogenates were incubated in parallel at pH7.7 at 37°C for 30min. Treatment of the mixtures with diazotized ethyl anthranilate and t.l.c. of the derived azopigments indicated formation of conjugated bilirubin in the controls. This amounted to 20% of the values found with the test mixtures (Mg^{2+} and UDP-glucuronic acid present). Therefore controls containing unactivated homogenate (and presumably also

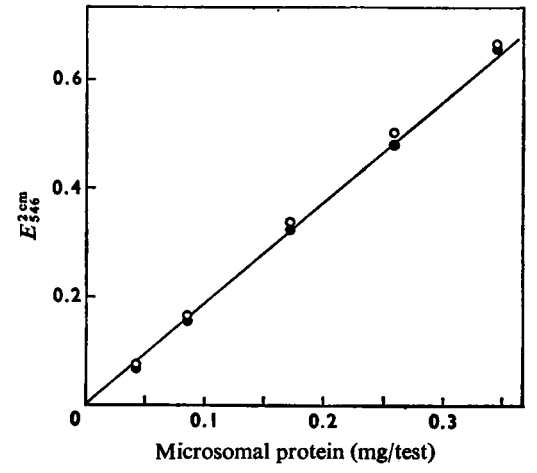

Fig. 4. *Effect of the amount of microsomal material on the activity of bilirubin UDP-glucuronyltransferase*

Mixtures (0.2ml) of washed microsomal material and digitonin were kept at 0°C for 30min (●). They contained 5, 10, 20, 30 and 40mg wet wt. equiv. of liver, and 0.135, 0.270, 0.540, 0.810 and 1.08mg of digitonin respectively (the ratio of both components was kept constant in the mixtures). The preincubated samples were then assayed as described in the Materials and Methods section. In parallel a larger volume with the same composition as the most concentrated one mentioned above was kept at 0°C for 30min (○). Dilutions were then prepared with 0.25M-sucrose containing 1mM-disodium EDTA, pH7.4, and assayed as above.

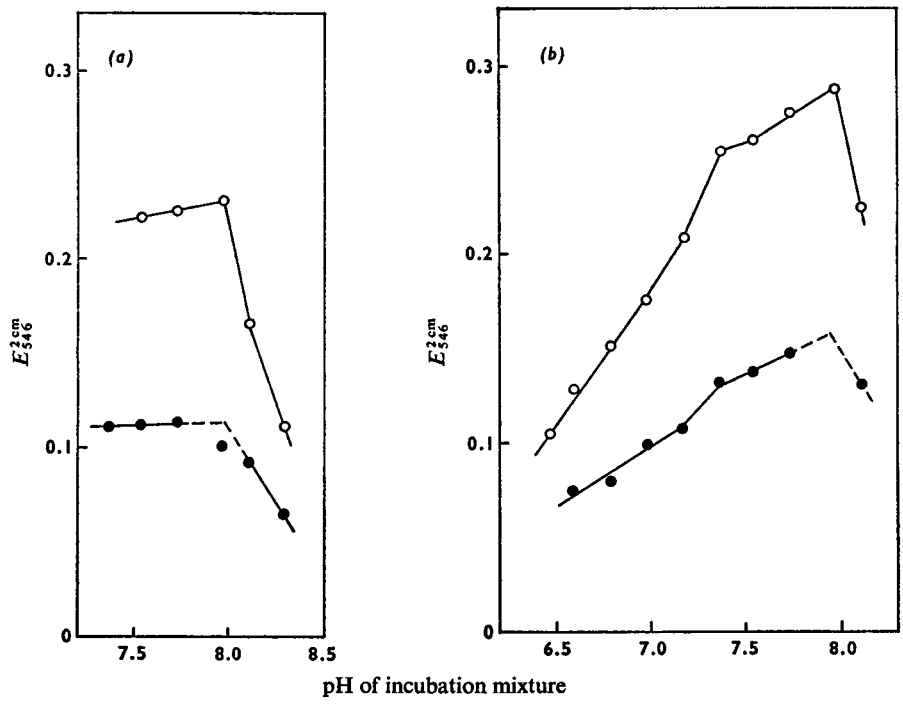

Fig. 5. *pH–activity curves*

Washed microsomal preparations were activated with digitonin and assayed as described in the Materials and Methods section. Incubation was (*a*) at 25°C for 15 min (●) and for 30 min (○), and (*b*) at 37°C for 5 min (●) and for 10 min (○). The triethanolamine concentration of the standard triethanolamine–HCl buffer was varied to obtain the final pH values. Duplicate incubation mixtures (UDP-glucuronic acid omitted) served for determination of pH.

cell extract) should preferably be kept at 0°C during the enzymic incubation stage, as done by Van Roy & Heirwegh (1968). However, with untreated microsomal preparations and with digitonin-activated homogenates and derived cell fractions no endogenous synthesis could be detected. Thus incubation of controls at 37°C is permissible. Condition (2) is expected to be fulfilled at least as well with the present system as with the earlier version of the method (Van Roy & Heirwegh, 1968) (incubation of controls at 0°C), as any change of interfering pigment (whether diazo-positive or not) should presumably be the same.

When tested on 12 microsomal preparations control values averaged $E^{2cm}_{546} = 0.059 \pm 0.13$ (S.D.). Colour was mainly due to bilirubin (Fig. 1*a*) with relatively small contributions from the enzyme preparations. Values obtained at increasing concentrations of activated homogenate are shown in Fig. 1(*b*).

Enzyme activity

Activation with digitonin. The treatment is based on the work of Pogell & Leloir (1961). With mixtures of microsomal material transfer of glucuronic acid was maximal at digitonin concentrations of 2–3 mg/ml when the microsomal concentration was 0.1 g wet wt. equiv. of liver/ml of suspension (1.9–2.6 mg of microsomal protein/ml) (six experiments; see, e.g., Fig. 2). Prolonged treatment at various digitonin concentrations (four experiments) resulted in almost identical maximal activities (compared with the levels reached with the standard system) with an extension of the zone of maximal activity to lower digitonin concentrations (Fig. 3).

In a similar way the optimum digitonin concentration was found to be 6 mg/ml with mixtures of digitonin and cell extract (0.125 g wet wt. equiv. of liver/ml of mixture). Without further control the same conditions have been adopted for work with liver homogenate.

Table 1. *Activity of bilirubin UDP-glucuronyltransferase in untreated and in digitonin-activated homogenates, cell extracts and washed microsomal preparations from rat liver*

Duplicate assay mixtures were measured at different times in the constant portion of the velocity–time curve (see the Materials and Methods section). Results are expressed as mean values±S.D. for the numbers of preparations given in parentheses. Recovery of activity is expressed as the percentage of the value found with cell extract.

Source of enzyme	Bilirubin conjugated (nmol/10min per mg of protein)	Bilirubin conjugated (nmol/10min per g wet wt. equiv. of liver)	Ratio of activity in activated/activity in untreated material	Recovery of activity (%)
Homogenate				
Untreated	0.30±0.05 (6)	55±10 (6)		
Activated	4.24±0.63 (6)	731±116 (6)	14±3 (6)	
Cell extract				
Untreated	0.45±0.04 (6)	53±6 (6)		100
Activated	5.56±0.89 (6)	640±104 (6)	12±1 (6)	100
Washed microsomal preparations				
Untreated	1.49±0.21 (6)	13±3 (6)		24±6 (6)
Activated	17.1±4.0 (5)	138±25 (5)	11±3 (5)	22±1 (5)

When activated under optimum conditions, homogenates, cell extracts and microsomal preparations were about 12-fold more active than untreated control preparations (Table 1). Under-estimation of the activities of untreated homogenates (see the preceding paragraph) probably explains the higher activation ratios found with homogenates.

Effect of microsomal protein. Enzyme activity was proportional to the amount of microsomal protein (Fig. 4). Activation of a concentrated suspension followed by the preparation of dilutions before the assays (Fig. 4, open circles) or activation of corresponding dilutions (filled-in circles) led to identical results.

Incubation time. With microsomal preparations and cell extracts reaction velocities were constant for about 20min (Figs. 6, 7 and 9).

Effect of pH. At 37°C enzyme activity of microsomal preparations was maximal at pH7.96 and declined slowly towards pH7.4 (two experiments; see, e.g., Fig. 5*b*). The behaviour at 30°C (results not shown) and at 25°C (Fig. 5*a*) was similar. Deviation from initial velocities was minimal in the near-plateau region. With a purified preparation from rat liver, Halac & Reff (1967) found maximal activity at pH7.8–8.0.

As the optimum pH is close to the steeply descending limb of the activity–pH curve a somewhat lower value (pH7.7) was selected for developing the standard assay. Unactivated enzyme activities (Table 1) were determined at the same pH value. Compared with the procedure of Van Roy & Heirwegh (1968) the volume of buffer used in the standard system was increased to give easier control of pH. This, of course, necessitated an increase in the volume of the glycine–HCl buffer (added at the colour-formation stage) to give a pH of about 2.7 (Van Roy & Heirwegh, 1968).

Incubation temperature. To facilitate eventual adoption of lower incubation temperatures a few assays were run at 25°, 30° and 37°C (three experiments). Conjugation velocity increased about threefold when the temperature was increased by 10°C. No break was apparent when the logarithm of the transfer rate was plotted against the reciprocal of the absolute temperature.

Stability of UDP-glucuronic acid

To test the stability of the sugar nucleotide, incubation mixtures containing fully activated microsomal material and all standard components except bilirubin were shaken for 30–120min at 37°C. After the addition of trichloroacetic acid P_i was determined in the supernatant solutions. The molar ratio of the liberated P_i to the UDP-glucuronic acid present at zero time was 0.034 after 30min and 0.16 after 120min incubation. Identical results were obtained when the human serum albumin in the mixtures was replaced by sucrose.

No tests were made with added 5′-nucleotidase. Therefore conversion of UMP (produced by UDP-glucuronic acid pyrophosphatase) into P_i could have been rate-limiting. Over the standard incubation periods (5–20min) phosphate formation relative to UDP-glucuronic acid is obviously small. Even if more UDP-glucuronic acid had been broken down than the amount of P_i released the effect on the observed conjugation rates appears to be negligible. Indeed, addition of 2.9mM-UDP-*N*-acetylglucosamine, a

Table 2. *Effect of UDP-N-acetylglucosamine on glucuronide formation*

Digitonin-activated homogenate and microsomal preparations (digitonin-activated or not) were used. Paired test and control incubation mixtures, containing the usual components and either 20 μl of water (standard system) or 20 μl of 100 mM-UDP-*N*-acetylglucosamine were assayed (see the Materials and Methods section). Results are expressed as nmol of bilirubin conjugated/10 min per g wet wt. equiv. of liver.

		Glucuronide formation	
Enzyme preparation	Treatment	Standard system	Standard system +UDP-*N*-acetyl-glucosamine
Washed microsomal preparation no. 1	Untreated	11.0	42.2
	Digitonin-activated	158	164
Washed microsomal preparation no. 2	Untreated	11.0	42.3
	Digitonin-activated	152	160
Washed microsomal preparation no. 3	Untreated	10.8	44.3
	Digitonin-activated	168	169
Homogenate	Digitonin-activated	900	785

known inhibitor of the pyrophosphatase (Pogell & Leloir, 1961; Adlard & Lathe, 1970), had little effect on the conjugation rates of activated microsomal material (Table 2). Similar observations have been made with Triton X-100-activated *p*-nitrophenyl UDP-glucuronyltransferase (Winsnes, 1969) and with purified bilirubin UDP-glucuronyltransferase (Adlard & Lathe, 1970). In contrast, as also reported by Adlard & Lathe (1970), conjugation rates of untreated microsomal preparations were increased considerably by the presence of the acetylamino sugar nucleotide (Table 2).

Variation of bilirubin and UDP-glucuronic acid concentrations

Bilirubin concentration. Deviation from Michaelis–Menten kinetics was noted (Fig. 6). At high concentrations substrate inhibition was apparent (Tomlinson & Yaffé, 1966; Adlard & Lathe, 1970). Deviation at low concentrations may be related to non-proportionality of total and unbound bilirubin (the binding protein being kept at constant concentration in the assays) (Adlard & Lathe, 1970).

In establishing the standard assay system a bilirubin concentration (0.12 mM) slightly below the optimum value but well removed from the substrate-inhibition zone was selected. The molar ratio of bilirubin to albumin was about 1. Previously (Van Roy & Heirwegh, 1968) a ratio of about 2 was adopted, based on the assumption that two strong binding sites are present on serum albumin. Work by Jacobsen (1969) indicates that the first binding site has much greater affinity for bilirubin than any other sites.

UDP-glucuronic acid concentration. Double-reciprocal plots of activity against concentration were linear (Figs. 7 and 8). Substrate inhibition occurred

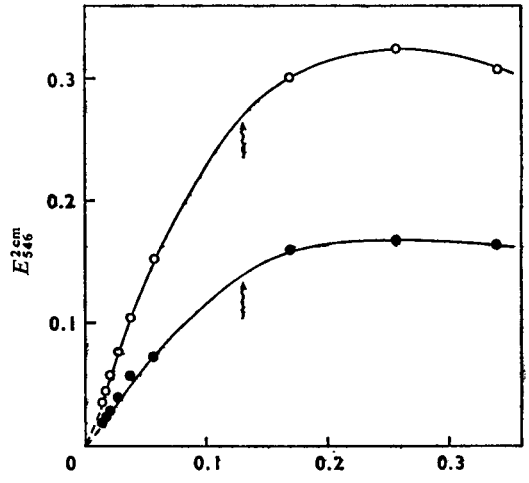

Fig. 6. *Effect of the concentration of the acceptor substrate, bilirubin*

A washed microsomal preparation was activated with digitonin and assayed as described in the Materials and Methods section. The enzymic incubation times were 10 min (●) and 20 min (○). The arrows indicate the standard bilirubin concentration.

at relatively high concentrations and was most pronounced at short incubation times (Fig. 8). Apparent K_m values (mean 0.37 mM) remained unchanged when the Mg^{2+} concentration was lowered to one-eighth of the standard concentration (Fig. 8; Table 3).

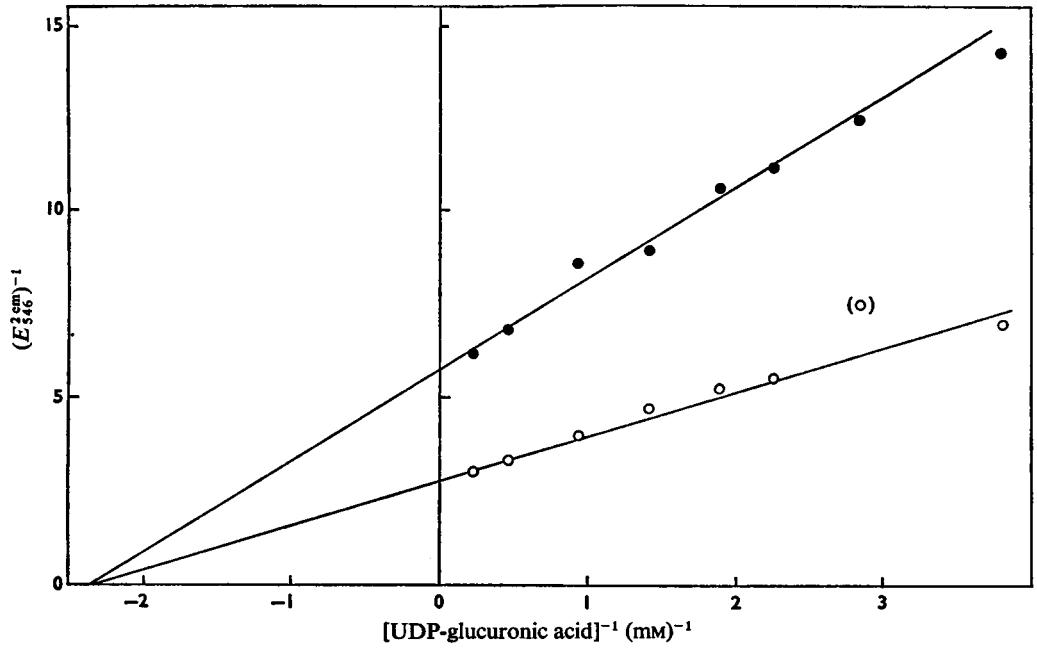

Fig. 7. *Double-reciprocal plots of enzyme activity against the concentration of UDP-glucuronic acid*

A washed microsomal preparation was activated with digitonin and assayed as described in the Materials and Methods section. The enzymic incubation times were 10min (●) and 20min (○). The experimental point in parenthesis was not taken into account to draw the corresponding straight line.

Activation by bivalent cations

Mg^{2+} stimulated glucuronic acid transfer with digitonin-treated microsomal preparations (Fig. 9). The curves suggested a partial requirement only. This is supported by incubation at increasing concentrations of disodium EDTA (Table 4).

Double-reciprocal plots of (total activity minus activity found in the absence of added Mg^{2+}) against the concentration of added Mg^{2+} were linear (Fig. 10). Apparent K_m values remained unchanged when the concentration of UDP-glucuronic acid was lowered to about one-fifth of the standard concentration (Table 3). In standard conditions substrate inhibition was noted from 10mM-Mg^{2+} onwards. In comparative experiments (not shown) inhibition started at lower concentrations (6–10mM-Mg^{2+}) when either the incubation time was decreased (10min instead of 20min) or when the UDP-glucuronic acid concentration was lower than its standard value.

Ca^{2+} was slightly less, and Mn^{2+} somewhat more, stimulatory than Mg^{2+} (Table 5). Establishment of a saturation curve with a microsomal preparation (enzymic incubation time 15min) demonstrated nearly constant maximal activities between 0.96 and 1.94mM-Mn^{2+}, with still 94 and 91% of the maximum activity at 0.64 and 5.6mM-Mn^{2+} respectively. Smaller stimulatory effects (compared with the enzyme activities found in the absence of added bivalent cation) were observed with Co^{2+}, Cd^{2+}, Fe^{2+} and Pb^{2+}; Zn^{2+} was inhibitory. In all cases transfer of glucuronic acid was demonstrated unequivocally by t.l.c. of the final azopigment extracts.

Recovery of bilirubin UDP-glucuronyltransferase activity

Activities when expressed on a liver-weight basis were comparable for homogenates and cell extracts (Table 1). Of the activities found in cell extracts about 23% was recovered in the microsomal preparations. Occasional checks indicated that a rather large percentage of the activity sedimented with the large-organelle fraction (7min at 41000$g_{av.}$), with smaller losses owing to washing of the microsomal preparations.

Discussion

The present assay system represents a development from the incubation system I of Van Roy & Heirwegh (1968), which uses albumin-bound bilirubin as the

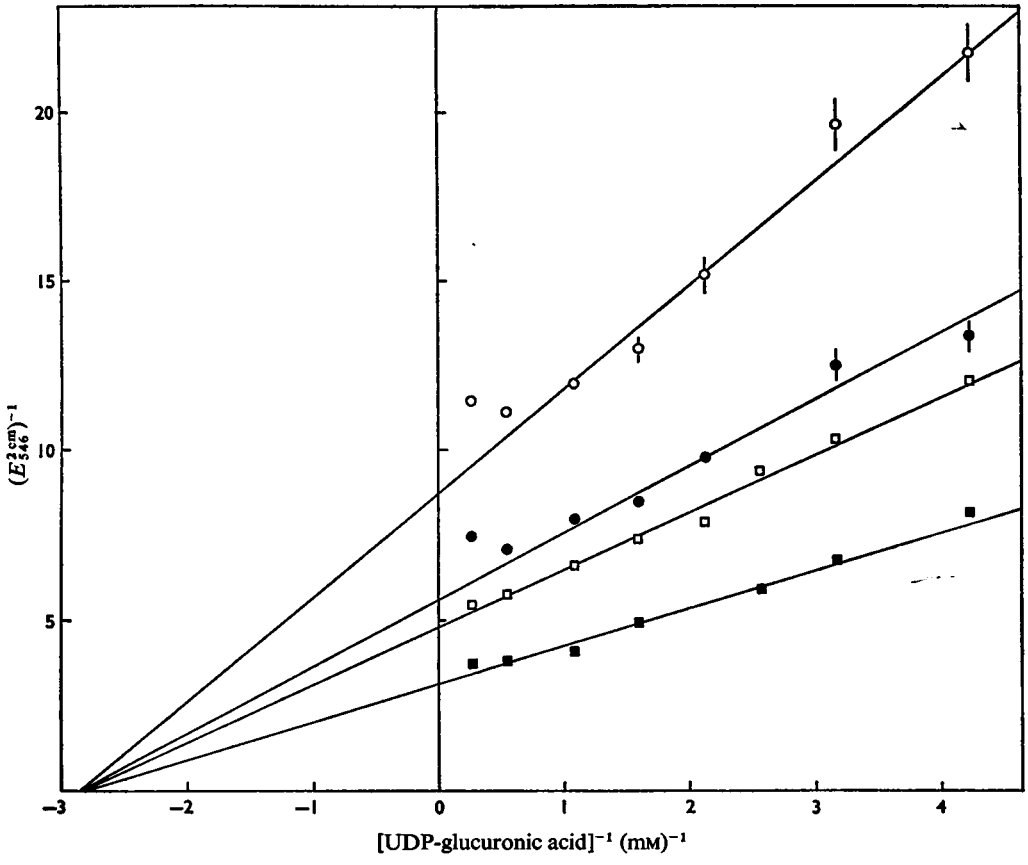

Fig. 8. *Double-reciprocal plots of enzyme activity against the concentration of UDP-glucuronic acid at two concentrations of Mg^{2+}*

A washed microsomal preparation (preparation no. 2 in Table 3) was activated with digitonin and assayed as described in the Materials and Methods section. Incubation times were 10min (○, ●) and 20min (□, ■). The final Mg^{2+} concentrations in the incubation mixtures were 0.9mM (○, □) and 7.2mM (●, ■). Vertical bars indicate minimal ranges of experimental error corresponding to a photometric uncertainty equal to ±0.002 on ($E_{test}-E_{blank}$). Details of the calculations are given by Fevery *et al.* (1972*a*).

acceptor substance. The more important modifications were (1) the development of more efficient procedures for extracting the azopigment derivatives and (2) the activation of the enzyme preparations with digitonin.

The adoption of the present extraction procedure is based on the following observations: (*a*) very energetic mixing with the concomitant formation of finely dispersed emulsions was found to lead, after settling, to clear aqueous and organic layers and to a very small, nearly colourless protein pellet; (*b*) settling was favoured by cooling.

Pretreatment of the enzyme preparations with digitonin led to an approx. 12-fold increase in activity (Figs. 2 and 3; Table 1). Activities expressed per unit wt. of protein or of liver were higher than any values obtained previously with albumin-bound bilirubin (Table 6). Activation may yield more consistent results. Indeed, when the early phase of the present work was concluded (Heirwegh & Meuwissen, 1968) unactivated enzyme activity of microsomal preparations was 2.8±0.6 (S.D.) nmol of bilirubin conjugated/10 min per mg of protein (13 preparations tested). This is significantly higher than the value obtained when the work was resumed (Table 1). However, the activated enzyme activity, 18.7±2.6 (S.D.; $n = 12$), was close

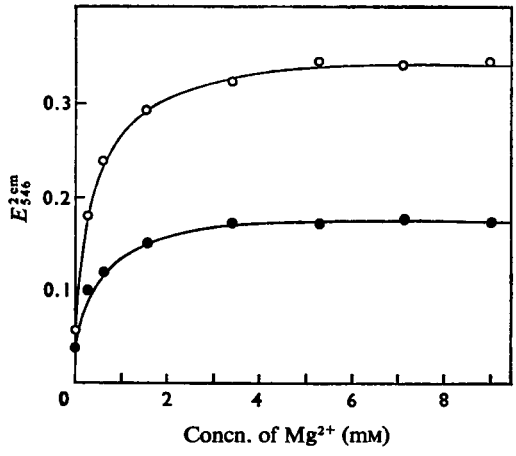

Fig. 9. *Activity of bilirubin UDP-glucuronyltransferase at increasing concentrations of* Mg^{2+}

A washed microsomal preparation was activated with digitonin and assayed as described in the Materials and Methods section. The following modifications were adopted: (1) the microsomal sediment was washed with 0.25 M-sucrose instead of sucrose–EDTA medium; (2) at all further stages disodium EDTA was omitted; (3) the enzyme activity at zero Mg^{2+} concentration was obtained by assaying the enzyme at a final disodium EDTA concentration of 0.9 mM. Enzymic incubation times were 10 min (●) and 20 min (○).

to the value found subsequently (Table 1). Uncontrollable activation (see the introduction for references) probably explains the wide range of reported enzyme activities (Table 6).

The choice of digitonin rather than a synthetic detergent may offer some advantages. Prolonged pretreatment at high concentrations did not produce any time-dependent harmful effects (Fig. 3). Compared with synthetic detergents (Halac & Reff, 1967; Winsnes, 1969), digitonin caused relatively minor inhibition at high concentrations (Figs. 2 and 3). Enzyme activity of fully activated preparations increased linearly with protein concentration, final values being determined solely by the digitonin/protein ratio (Fig. 4). Therefore some versatility in the choice of the ratio is possible by proper selection of the activation time (Fig. 3; see also Fevery *et al.*, 1972*a*).

Michaelis–Menten kinetics were followed with respect to UDP-glucuronic acid (Figs. 7 and 8), confirming work of Adlard & Lathe (1970) and of several other authors. However, studies by Winsnes (1971) suggest that binding of UDP-glucuronic acid to UDP-glucuronyltransferase is under allosteric control.

In accordance with previous work with rat liver preparations Mg^{2+} (Van Roy & Heirwegh, 1968; Adlard & Lathe, 1970; Frei, 1970; Mowat & Arias, 1970; Wong, 1971) and Ca^{2+} (Adlard & Lathe, 1970; Frei, 1970) stimulated glucuronyl transfer with digitonin-activated microsomal preparations (Fig. 9; Tables 4 and 6). However, in contrast with the work of Adlard & Lathe (1970), who demonstrated an absolute requirement for Mg^{2+} with a purified preparation from the liver of the hooded rat, part of the enzyme in digitonin-activated microsomal material from the liver of the Wistar rat functioned independently of added bivalent metal ion (Fig. 9; Table 4). Observations by Lage & Spratt (1968) suggest complete independence with microsomal preparations from guinea-pig liver. Glucuronyl transfer to other acceptor substances may or may not require the presence of Mg^{2+}, depending on the compound tested (Dutton, 1966).

The stimulatory effect of Mn^{2+} (Table 5) is not unexpected. Frequently Mn^{2+} can replace Mg^{2+} as an activator of enzymes acting on phosphorylated substrates (Dixon & Webb, 1965). Also optimum activation with Mn^{2+} generally occurs at lower concentrations. The effects of Mn^{2+}, Ca^{2+} and Zn^{2+} are in agreement with the work of Lucier *et al.* (1971) on the catalysis of glucuronyl transfer to *p*-nitrophenol and 1-naphthol by Triton X-100-activated and untreated microsomal preparations from the livers of rat and rabbit. Stimulation of UDP-glucuronyltransferase by Cd^{2+}, Fe^{2+} or Pb^{2+} (Table 5) has not been observed previously.

No satisfactory explanation of the conflicting metal-ion requirements of UDP-glucuronyltransferase and of the existence of a Mg^{2+}-independent enzyme fraction can be offered. Three problems may have to be solved first. During recent years several attempts have been made to solubilize UDP-glucuronyltransferase (e.g. Adlard & Lathe, 1970; Mowat & Arias, 1970). It appears to be doubtful, however, if any truly soluble preparation has been achieved (Attwood *et al.*, 1971). Next, the long-standing question of the possible heterogeneity of UDP-glucuronyltransferases has not been settled completely (for a review, see Dutton, 1971). Finally, observations on the endogenous synthesis of steroid glucosides (Williamson *et al.*, 1971) and of glucosides and glucuronosides of bilirubin (J. Fevery & K. P. M. Heirwegh, unpublished work) suggest that glycosyl transfer occurs over at least two enzymically catalysed reaction steps. Behrens & Leloir (1970) explained glucosyl transfer from UDP-glucose to an endogenous glycoprotein acceptor by a similar mechanism. It may be noted that only the first step, the transfer of glucose to dolichol monophosphate, showed a requirement for bivalent metal ion. Resolu-

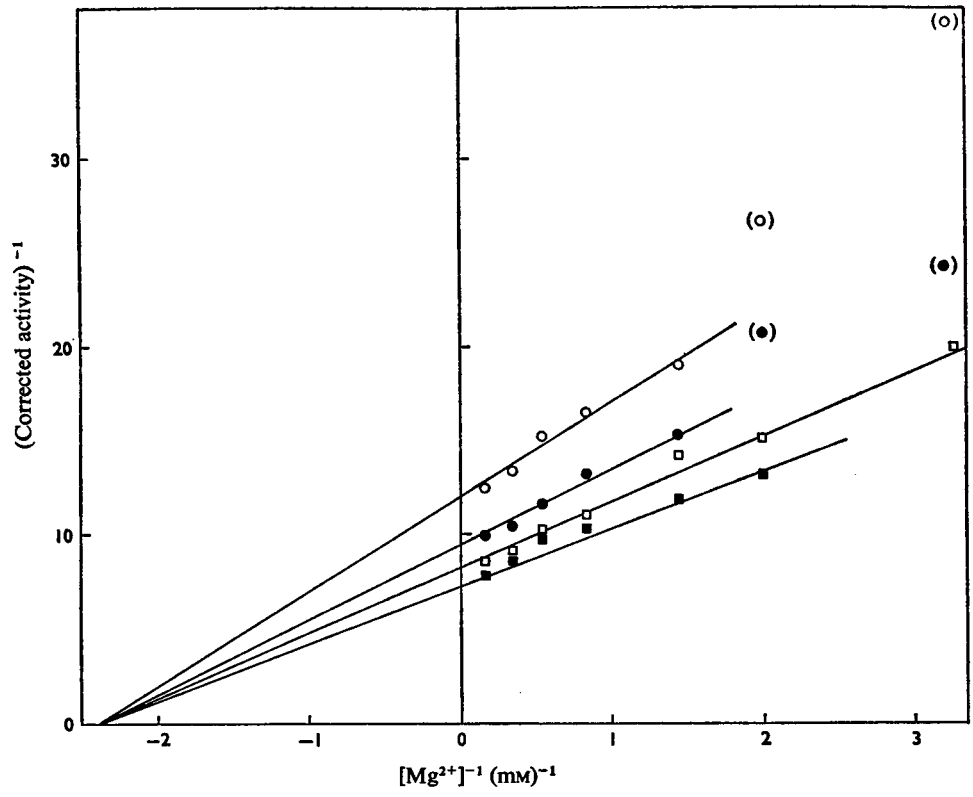

Fig. 10. *Double-reciprocal plots of corrected enzyme activity* ($E^{2cm}_{546} - E^{2cm}_{546}$ *found in the absence of added* Mg^{2+}) *against the concentration of added* Mg^{2+}

A washed microsomal preparation was activated with digitonin and assayed as described in the Materials and Methods section. The enzymic incubation time was 15min. Final concentrations of UDP-glucuronic acid in the incubation mixtures were 0.45mM (o), 0.98mM (•), 1.41mM (□) and 1.89mM (■). Experimental points in parentheses were not taken into account to obtain the apparent K_m values (see Table 3).

tion of the mechanisms of glycosyl transfer to hydrophobic acceptor substances may perhaps lead to a better understanding of the problems raised by metal-ion activation of UDP-glycosyltransferases.

Constancy of K_m for Mg^{2+} (related to the Mg^{2+}-dependent enzyme fraction) and of K_m for UDP-glucuronic acid, at several concentrations of the other substrate (bilirubin being at its standard concentration) (Table 3) suggests that K_m for Mg^{2+} represents the dissociation constant of an enzyme–Mg^{2+} complex (Dixon & Webb, 1965). The apparent Mg^{2+}-independent enzyme fraction (K_m for Mg^{2+}, 0.4mM; Table 3) cannot be explained by freely soluble endogenous Mg^{2+}. Indeed, according to results of Carvalho *et al.* (1965) and of Murdoch & Heaton (1968), the endogenous Mg^{2+} concentration in our incubation mixtures containing digitonin-activated microsomal material cannot be higher than 0.007mM. The Ca^{2+} concentration would be 0.015mM. Obviously, in the presence of 7.6mM-disodium EDTA, pH 7.7 (Table 4), the uncomplexed concentrations of both ions should be very small. It is suggested that the bivalent ion-independent UDP-glucuronyltransferase is a metalloprotein containing its share of activating ion in strongly bound form. It may be noted that the lipoprotein fraction of microsomal material has a strong tendency to bind Ca^{2+} and Mg^{2+} (Carvalho *et al.*, 1965). The existence of truly metal ion-independent enzyme is possible but less likely.

In view of the incomplete understanding of the UDP-glucuronyltransferase enzyme system(s) no preference can be given to the determination of activated or unactivated enzyme activities for the interpretation of metabolic changes.

Table 3. *Apparent K_m values of digitonin-activated bilirubin UDP-glucuronyltransferase with respect to UDP-glucuronic acid and Mg^{2+}*

Microsomal preparations were assayed with bilirubin at its standard concentration (see the Materials and Methods section). Apparent K_m values were obtained by application of the method of least squares to $1/v$ plotted as a function of 1/[substrate]. Velocity results affected by substrate inhibition were rejected.

Microsomal preparation no.	Incubation time (min)	Substrate at constant concentration		Substrate at varying concentration		
		Nature	Concn. (mM)	Nature	Range of concn. used (mM)	K_m (mM)
1	10	Mg^{2+}	7.2	UDP-glucuronic acid	0.26–4.2	0.38
	20		7.2		0.26–4.2	0.39
2	10		7.2		0.54–4.3	0.31
	10		0.9		0.54–4.3	0.41
	20		7.2		0.54–4.3	0.36
	20		0.9		0.54–4.3	0.37
3	10	UDP-glucuronic acid	2.0	Mg^{2+}	0.26–3.4	0.30
	20		2.0		0.26–3.4	0.35
4	12		2.1		0.35–3.5	0.44
5	15		1.9		0.57–5.7	0.40
	15		1.4		0.38–5.7	0.45
	15		1.0		0.76–5.7	0.47
	15		0.45		0.76–5.7	0.42

Table 4. *Effect of disodium EDTA on digitonin-activated bilirubin UDP-glucuronyltransferase*

By using the standard assay system (see the Materials and Methods section) microsomal preparations were assayed (*a*) at 7.6mM-Mg^{2+}, (*b*) with omission of Mg^{2+} and (*c*) with replacement of Mg^{2+} by increasing concentrations of disodium EDTA, pH7.7. Activities are expressed as percentages of the values found in the presence of Mg^{2+}. Tests were run at 0.35–2.1mM-UDP-glucuronic acid.

		Relative activity									
Preparation no.	...	1	1	2	2	3	3	3	3	4	4
Final concn. of UDP-glucuronic acid (mM)	...	2.0	2.0	2.1	0.35	1.9	1.4	1.0	0.45	2.0	2.0
Incubation time (min)	...	10	20	12	12	15	15	15	15	10	20
Addition	Concn. (mM)										
Mg^{2+}	7.6	100	100	100	100	100	100	100	100	100	100
None				28	23	32	29	28	27	16	20
EDTA	0.76			29	31						
	1.24	39	27								
	3.80			35	31						
	7.60			33	29					19	17

However, apart from practical advantages mentioned above some justification for assaying previously activated bilirubin UDP-glucuronyltransferase and related UDP-glycosyltransferases (Fevery *et al.*, 1972*a*) may be found in considering discordant results relating to the study of bilirubin metabolism. At increasing concentrations of infused bilirubin, secretion of bilirubin conjugates into rat bile is rate-limiting, saturation of the secretory apparatus leading to return of conjugated bilirubin into the blood (Schmid *et al.*, 1958; Arias *et al.*, 1961). As normal rat bile contains mainly the mono- and di-glucuronoside of bilirubin (Schoenfield & Bollman, 1963; Ostrow & Murphy, 1970; Heirwegh *et al.*, 1970) one would

Table 5. *Effects of bivalent metal ions on bilirubin UDP-glucuronyltransferase*

Digitonin-activated cell extract and microsomal material were assayed in parallel in the presence (*a*) and in the absence (*b*) of bivalent cation. The assay systems described in the Materials and Methods section were used except for complete omission of disodium EDTA from the incubation mixtures. Enzyme activities are given as percentages of the values found at 5.77mM-Mg^{2+} (taken as 100%). Individual values are indicated when more than one preparation was tested.

	Source of enzyme ...	Microsomal preparation		Cell extract	
Addition	Concn. of added material (mM) ...	0.99	5.77	0.91	5.77
(*a*) Mg^{2+}			100		100
Ca^{2+}		63, 64	91, 91		
Ba^{2+}		17	23		
Zn^{2+}				22	2
Cd^{2+}		23	35		
Pb^{2+}		37	65		
Mn^{2+}		102, 110, 117	95, 109	111	92
Fe^{2+}		24, 40	42, 70		
Co^{2+}		54, 55	55, 66	57	47
Ni^{2+}				55	41
(*b*) None		17, 22, 24			

Table 6. *Bilirubin UDP-glucuronyltransferase activity in the preparation from rat liver*

Only results obtained with incubation systems containing pure (or purified) UDP-glucuronic acid and albumin-solubilized bilirubin are given. Activities are expressed as nmol of bilirubin conjugated/10min per mg of protein (A_P) or as nmol of bilirubin conjugated/10min per g wet wt. equiv. of liver (A_L).

Source	Enzyme preparation pretreatment	Activity A_P	Activity A_L	Rat strain and sex	Reference
Slices	None		16	Wistar	Lathe & Walker (1958)
	None		9		Hargreaves *et al.* (1969)
	None		28	Hooded, male	Adlard & Lathe (1971)
Homogenates	None		69–82	Sprague–Dawley, male	Metge *et al.* (1964)
	None		33	Sprague–Dawley	DeLeon *et al.* (1967)
	None		48	Sprague–Dawley	Halac & Reff (1967)
	None	0.60		Sprague–Dawley	Mowat & Arias (1970)
	None	0.80		Wistar	Mowat & Arias (1970)
	None	0.30	55	Wistar R, albino, male	Present paper
	Purified		380	Sprague–Dawley	Halac & Reff (1967)
	Digitonin-treated		425	Sprague–Dawley	Black *et al.* (1970)
	Digitonin-treated	4.2	731	Wistar R, albino, male	Present paper
Microsomal preparations	None		43–83	Wistar, male	Menken *et al.* (1966)
	None		48	Wistar R, albino, male	Van Roy & Heirwegh (1968)
	None	0.6–0.9		Hooded, male	Adlard & Lathe (1970)
	None	1.5	13	Wistar R, albino, male	Present paper
	Purified	5.0		Wistar	Mowat & Arias (1970)
	Purified	6.0		Sprague–Dawley	Mowat & Arias (1970)
	Purified	8.2–14		Hooded, male	Adlard & Lathe (1970)
	Digitonin-treated	17	138	Wistar R, albino, male	Present paper

expect the activity of bilirubin UDP-glucuronyltransferase to be larger than the maximal excretion rate at 37°C, 600nmol of conjugated bilirubin/10min per g of liver (Van Damme & Desmet, 1969). This is indeed the case for fully activated enzyme (Table 1). Further, in contrast to the nearly negligible excretion

in rat bile of glucose and xylose conjugates of bilirubin compared with the glucuronosides (Fevery *et al.*, 1971), untreated rat liver homogenate showed transfer rates from either UDP-glucose or UDP-xylose to bilirubin about twofold that from UDP-glucuronic acid (Fevery *et al.*, 1972*a*). After digitonin activation the proportion was approximately reversed.

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