Enzymic Transfer of Glucose and Xylose from Uridine Diphosphate Glucose and Uridine Diphosphate Xylose to Bilirubin by Untreated and Digitonin-Activated Preparations from Rat Liver

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(Received 23 February 1972)

1. Digitonin-treated and untreated homogenates, cell extracts and washed microsomal preparations from liver of Wistar R rats are capable of transferring sugar from UDP-glucose or UDP-xylose to bilirubin. No formation of bilirubin glycosides occurred with UDP-galactose or D-glucose, D-xylose or D-glucuronic acid as the sources of sugar. 2. Procedures to assay digitonin-activated and unactivated bilirubin UDP-glucosyltransferase and bilirubin UDP-xylosyltransferase were developed. 3. In digitonin-activated microsomal preparations the transferring enzymes had the following properties. Both enzyme activities were increased 2.5-fold by pretreatment with digitonin. They were optimum at pH 6.6–7.2. Michaelis–Menten kinetics were followed with respect to UDP-glucose. In contrast, double-reciprocal plots of enzyme activity against the concentration of UDP-xylose showed two intersecting straight-line sections corresponding to concentration ranges where either bilirubin monoxyside was formed (at low UDP-xylose concentrations) or where mixtures of both the mono- and di-xyside were synthesized (at high UDP-xylose concentrations). Both enzyme activities were stimulated by Mg^{2+} ; Ca^{2+} was slightly less, and Mn^{2+} slightly more, stimulatory than Mg^{2+} . Of the activities found in standard assay systems containing Mg^{2+} , 58–78% (substrate UDP-glucose) and 0–38% (substrate UDP-xylose) were independent of added bivalent metal ion. Double-reciprocal plots of the Mg^{2+} -dependent activities against the concentration of added Mg^{2+} were linear. 4. In comparative experiments the relative activities of liver homogenates obtained with UDP-glucuronic acid, UDP-glucose and UDP-xylose were 1:1.5:2.7 for untreated preparations and 1:0.29:0.44 after activation with digitonin. 5. Bilirubin UDP-glucuronyltransferase was protected against denaturation by human serum albumin, whereas bilirubin UDP-xylosyltransferase was not. 6. Digitonin-treated and untreated liver homogenates from Gunn rats were inactive in transferring sugar to bilirubin from UDP-glucuronic acid (in agreement with the work of others), UDP-glucose or UDP-xylose.

As demonstrated by azopigment analysis, post-obstructive bile of man (Kuenzle, 1970; Heirwegh *et al.*, 1970) and rat (Van Damme *et al.*, 1971), and bile of the normal dog (Fevery *et al.*, 1971), show complex bile-pigment compositions. Several pathways seem therefore to be involved in the metabolism of the aglycone. From dog bile the β -D-monoglucopyranoside and the β -D-monoxypyranoside of azodipyrrole were obtained in appreciable amounts (Fevery *et al.*, 1971; Compernelle *et al.*, 1971), indicating that bilirubin conjugates of D-glucose and D-xylose must occur in bile. Much smaller amounts of azopigments moving chromatographically as the two substances mentioned above were obtained from human and from rat bile (Fevery *et al.*, 1971).

A search was made for enzyme systems conjugating bilirubin with either glucose or xylose. With UDP-

glucose or UDP-xylose as the sugar donors conjugation of bilirubin was observed with homogenates, cell extracts and microsomal preparations from the liver of rat. Properties of the transferring systems are described. A study of the reaction products is described in the next paper (Fevery *et al.*, 1972). A preliminary communication of the present investigations has been given (Heirwegh *et al.*, 1971).

Materials and Methods

Chemicals

Chemicals were as specified by Van Roy & Heirwegh (1968) and by Heirwegh *et al.* (1972) except for the following. UDP-glucose (sodium salt) was from E. Merck A.-G. (Darmstadt, Germany). UDP-galactose (sodium salt), and UDP-xylose (sodium

salt) were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Biological materials

Adult male Wistar R albino rats (body wt. 250–400 g) and adult male Gunn rats were fed on RMH-B food (Hope Farms, Woerden, The Netherlands) and had free access to drinking water. Gunn rats (*jj*), characterized by hereditary unconjugated hyperbilirubinaemia, are derived from the Wistar rat (*JJ*) by mutation. This characteristic is transferred as an autosomal, recessive trait, and is related to the partial or total inability of the mutant animal to conjugate endogenous and exogenous compounds with glucuronic acid (see, e.g., Schmid, 1966).

Liver homogenates and cell fractions from rat liver were obtained by the method of de Duve *et al.* (1955) as described by Heirwegh *et al.* (1972). As a diluent 0.25 M-sucrose containing 1 mM-disodium EDTA, pH 7.4, was used except when stated otherwise.

Assays of bilirubin UDP-xylosyltransferase and bilirubin UDP-glucosyltransferase activities

The procedure of Heirwegh *et al.* (1972) was followed except for the conditions of activation with digitonin and for minor changes in the compositions of the incubation mixtures.

Digitonin-activated enzyme preparations. Before the enzymic incubations were started, mixtures of the selected preparation and of digitonin were kept at 0°C for 30 min. These mixtures contained either (1) 1 vol. of homogenate or cell extract (0.125 g wet wt. equiv. of liver/ml of suspension for studies with UDP-xylose and 0.25 g/ml for work with UDP-glucose) and 1 vol. of digitonin suspension (12 mg/ml), or (2) 1 vol. of microsomal preparation (2 g wet wt. equiv. of liver/ml) and 1 vol. of digitonin suspension (16 mg/ml). Digitonin suspensions were prepared by heating briefly the required amounts of digitonin and sucrose-EDTA medium on a boiling-water bath.

Incubation mixtures. Components of incubation mixtures were: 200 μ l of triethanolamine-HCl buffer (0.5 M-HCl adjusted to pH 7.1 with triethanolamine), 30 μ l of 125 mM-MgCl₂ soln., 200 μ l of bilirubin-albumin mixture (prepared as described by Heirwegh *et al.*, 1972), 200 μ l of digitonin-activated enzyme preparation and either 20 μ l of UDP-xylose (54.5 mg/ml) or 100 μ l of UDP-glucose soln. (125 mg/ml). The components of the incubation mixtures were combined in the sequence given except when stated otherwise. All concentrations refer to reagents as used. Incubation was carried out at 37°C with shaking.

Untreated enzyme preparations. The procedure outlined above was followed but samples of homo-

genate (200 μ l) or cell extract (50 mg wet wt. equiv. of liver) or of a washed microsomal preparation (200 mg wet wt. equiv. of liver) replaced the digitonin-activated preparations.

Calculation of the minimal range of experimental error

In representing double-reciprocal plots in Figs. 4–8 estimates of the range of experimental error are indicated by vertical bars. Where not shown the bars are adsorbed by the dots. The calculations are based on the following assumptions. (1) The instrumental uncertainty inherent in photometric determination of test and blank values limits the total, final error. (2) The absolute error of the instrument used is $E \pm 0.001$. Such a procedure obviously yields minimal estimates because pipetting errors etc. are neglected. At low reaction rates assumption (1) is probably verified. For instance, when the value of ($E_{\text{test}} - E_{\text{blank}}$) is 0.050, then the instrumental error would be ± 0.002 and the relative error $\pm 4\%$. Contributions to the experimental error from pipetting etc. can usually be kept well below 1%.

Ancillary determinations

Digitonin-activated bilirubin UDP-glucuronyltransferase was assayed as described by Heirwegh *et al.* (1972). Bilirubin in bilirubin-albumin mixtures and in microsomal preparation assay systems devoid of UDP-sugar was determined as described by Van Roy *et al.* (1971). 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). The concentrations of aqueous solutions of UDP-sugars were determined by assuming ϵ_{261} 9.9×10^3 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ at neutral pH (Bock *et al.*, 1956).

Results

Except when explicit mention is made to the contrary (Table 7 and the last paragraph of the present section) all results refer to work with preparations from the livers of Wistar R rats. The results illustrated in the figures and in Tables 2–6 refer to work with digitonin-activated microsomal material.

Transferase activity

Activation with digitonin. At increasing digitonin concentration the activities of the transferases acting on UDP-glucose and UDP-xylose increased about 2.5-fold (Table 1). The shapes of the activation curves were similar to those found with UDP-glucuronic acid by Heirwegh *et al.* (1972).

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

The activities with UDP-glucuronic acid, UDP-glucose and UDP-xylose as the sugar donors were determined in parallel on the same enzyme preparations. Each result was obtained from two determinations at different time-points in the constant portions of the velocity-time curves. The assays are outlined in the Materials and Methods section; results for UDP-glucuronic acid are taken from Heirwegh <i>et al.</i> (1972). Results are expressed as mean values $\pm$ s.d.; the number of preparations tested is indicated in parentheses.														
Source of enzyme	Sugar donor	Bilirubin conjugated (nmol/10 min per mg of protein)			Bilirubin conjugated (nmol/10 min per g wet wt. of liver)			Ratio of activity in untreated/ activity in activated material			Recovery of enzyme in washed microsomal fractions (%)			
		UDP-glucose	UDP-xylose	UDP-glucuronic acid	UDP-glucose	UDP-xylose	UDP-glucuronic acid	UDP-glucose	UDP-xylose	UDP-glucuronic acid	UDP-glucose	UDP-xylose	UDP-glucuronic acid	
Homogenate	Untreated	0.49 $\pm$ 0.09 (3)	0.86 $\pm$ 0.19 (3)	0.30 $\pm$ 0.05 (6)	83 $\pm$ 22 (3)	149 $\pm$ 36 (3)	55 $\pm$ 10 (6)							
	Activated	1.24 $\pm$ 0.19 (3)	2.09 $\pm$ 0.28 (3)	4.24 $\pm$ 0.63 (6)	213 $\pm$ 37 (3)	318 $\pm$ 38 (3)	731 $\pm$ 116 (6)	2.2 $\pm$ 0.5 (3)	2.6 $\pm$ 0.6 (3)	14 $\pm$ 3 (6)				
Cell extract	Untreated	0.60 $\pm$ 0.04 (3)	1.16 $\pm$ 0.05 (3)	0.45 $\pm$ 0.04 (6)	66 $\pm$ 5 (3)	124 $\pm$ 8 (3)	53 $\pm$ 6 (6)				100	100	100	
	Activated	1.62 $\pm$ 0.21 (3)	2.50 $\pm$ 0.13 (3)	5.56 $\pm$ 0.89 (6)	183 $\pm$ 40 (3)	280 $\pm$ 20 (3)	640 $\pm$ 104 (6)	2.3 $\pm$ 0.2 (3)	2.7 $\pm$ 0.3 (3)	12 $\pm$ 1 (6)	100	100	100	
Washed microsomal preparations	Untreated	2.01 $\pm$ 0.19 (3)	2.96 $\pm$ 0.19 (3)	1.49 $\pm$ 0.21 (6)	18 $\pm$ 3 (3)	27 $\pm$ 5 (3)	13 $\pm$ 3 (6)				27 $\pm$ 2 (3)	22 $\pm$ 3 (3)	24 $\pm$ 6 (6)	
	Activated	5.07 $\pm$ 0.34 (3)	7.66 $\pm$ 0.34 (3)	17.1 $\pm$ 4.0 (5)	46 $\pm$ 9 (3)	69 $\pm$ 9 (3)	138 $\pm$ 25 (6)	2.6 $\pm$ 0.2 (3)	2.5 $\pm$ 0.3 (3)	11 $\pm$ 3 (5)	25 $\pm$ 3 (3)	25 $\pm$ 3 (3)	22 $\pm$ 1 (5)	

Fig. 1. Activities of bilirubin UDP-glucosyltransferase and of bilirubin UDP-xylosyltransferase as a function of the protein concentration

Two microsomal preparations were used (indicated by ○, ● and □ respectively). Mixtures of microsomal material (1.5 g wet wt. equiv. of liver/ml) and digitonin (12mg/ml) were kept at 0°C for 30min. Dilutions were then prepared with 0.25M-sucrose containing 1mM-disodium EDTA, pH7.4, and assayed with UDP-glucose (○, 12min; ●, 6min) or with UDP-xylose (□, 6min) at 6min and 12min incubation times.

Results obtained in parallel with UDP-glucuronic acid as the sugar donor, on the same enzyme preparations, are shown in Table 1. The enzyme activities, when expressed on a liver-weight basis, were roughly the same in homogenates and cell extracts, whether digitonin-activated or not. Similar fractions of the activities were recovered in the microsomal preparations (about 25% of the activities found in the cell extracts). In contrast, activation (2.5-fold) was much lower than that found with bilirubin UDP-glucuronyltransferase (about 12-fold). The relative activities of liver homogenate obtained with UDP-glucuronic acid, UDP-glucose and UDP-xylose were 1:1.5:2.7 for untreated preparations and 1:0.29:0.44 after activation with digitonin.

Effect of microsomal protein. The activities increased linearly with the concentration of microsomal protein when the ratio of microsomal material to digitonin was kept constant at the value adopted for the standard incubation system (Fig. 1).

Incubation time. With microsomal preparations transfer rates were constant for about 16min with

Fig. 2. pH-activity curves of (a) bilirubin UDP-xylosyltransferase and (b) bilirubin UDP-glucosyltransferase

Two microsomal preparations (indicated by $\circ$, $\bullet$ and $\square$ respectively) were assayed. The incubation time of the preparation denoted by $\square$ was 30 min. Duplicate assay mixtures were incubated for 5 min ($\bullet$) and 10 min ($\circ$) with UDP-xylose as the sugar donor (a) and for 8 min ($\bullet$) and 16 min ($\circ$) with UDP-glucose (b). Different pH values were obtained by varying the concentration of the triethanolamine used to prepare the standard buffer. Incubation mixtures prepared in parallel (UDP-sugar omitted) were used to determine the pH.

Table 2. Release of P_i during incubation of UDP-glucose and UDP-xylose in microsomal incubation systems

Microsomal incubation systems (see the Materials and Methods section) from which bilirubin had been omitted were shaken at 37°C. At zero time the concentrations of UDP-glucose and UDP-xylose were 27.7 and 2.54 mM respectively. After the indicated incubation periods trichloroacetic acid was added and P_i determined on the supernatants by the method of Fiske & Subbarow (1925). Values were corrected for endogenous P_i present in the systems at zero time. Release of P_i is expressed as the molar ratio of P_i found to UDP-sugar present at zero time.

Incubation time (min)	Ratio of P_i /UDP-sugar present at zero time	
	UDP-glucose	UDP-xylose
10	0.031	
20	0.057	
30	0.088	0.14
60	0.11	0.30
90	0.16	0.44
120	0.20	0.46

Fig. 3. Stability of bilirubin UDP-xylosyltransferase and bilirubin UDP-glucuronyltransferase

Portions of a microsomal preparation were activated with digitonin as appropriate for work with UDP-xylose ($\square$, $\blacksquare$) and UDP-glucuronic acid ($\circ$, $\bullet$). To 0.2 ml portions of the activated preparations triethanolamine-HCl buffer ($\square$, $\blacksquare$, pH 7.1; $\circ$, $\bullet$, pH 7.7), $MgCl_2$ soln. and albumin-bilirubin mixture ($\circ$, $\square$) were added (the volumes and compositions were as indicated in the Materials and Methods section). Duplicate mixtures from which the albumin-bilirubin mixture was omitted were also prepared ($\bullet$, $\blacksquare$). The resulting mixtures were then shaken at 37°C for the periods of time indicated along the abscissa. Next they were cooled to 0°C, completed with the appropriate UDP-sugar soln. and eventually with albumin-bilirubin mixture ($\bullet$, $\blacksquare$) and assayed as described in the Materials and Methods section. Portions of the initial mixtures of microsomal material and digitonin, kept at 0°C during the preincubations, served to prepare the zero-time controls (taken as 100%).

UDP-glucose as the sugar donor, and for about 12 min with UDP-xylose. In general, duplicate assay mixtures were run at different time-points situated in the constant portion of the velocity-time curves.

Effect of pH. Compared with work with UDP-glucuronic acid (Heirwegh *et al.*, 1972) more variable activity-pH curves were obtained (Fig. 2). With relatively short incubation times (5 min with UDP-xylose and 8 min with UDP-glucose) activities were approximately constant from pH 6.6 to 7.2, whereas the near-plateau region with UDP-glucuronic acid was situated at higher pH values (pH 7.4-7.96). At prolonged incubation (30 or 60 min) maxima at pH 6.6, and 7.1, or one maximum at pH 7.1 and a shoulder at pH 6.6, appeared. In all instances where UDP-glucose and UDP-xylose were tested in parallel with the same enzyme preparation, similarly shaped curves were found (five experiments; two examples are shown in Fig. 3).

Table 3. *Stability of digitonin-activated bilirubin UDP-xylosyltransferase and bilirubin UDP-glucuronyltransferase*

Suspensions of microsomal preparations (*A*, *B* and *C*) containing 2g wet wt. equiv. of liver/ml were treated at 4°C for 20h with equal volumes of digitonin suspension (20mg/ml) (complete activation was demonstrated by running standard enzyme assays on the initial untreated preparations and after 30min and 20h of activation). Samples of activated preparation (40 μ l for assays with UDP-glucuronic acid; 200 μ l for assays with UDP-xylose) were adjusted to pH 6.8, 7.1 or 7.6 with 40 μ l of triethanolamine-HCl buffer (0.5M-HCl adjusted to the required pH with triethanolamine) (*a*). In some experiments mixtures of type (*a*) were supplemented with 200 μ l of a solution of human serum albumin (21.6mg/ml) (*b*), or with 200 μ l of human serum albumin-bilirubin mixture (*c*) or with 30 μ l of 125mM-MgCl₂ soln. and 200 μ l of albumin-bilirubin mixture (*d*). The resulting mixtures were preincubated at 37°C for 30 or 60min. They were placed in ice-water and adjusted to the same composition as (*d*). Finally, 200 μ l of buffer, pH 7.1, and 20 μ l of UDP-xylose soln., or 200 μ l of buffer, pH 7.7, and 20 μ l of UDP-glucuronic acid soln., were added and the remaining enzyme activities were determined as described in the Materials and Methods section. Results are given as percentages of the activities found with duplicate mixtures (*a*, *b*, *c* and *d*) kept at 0°C during the preincubations.

Microsomal preparation	Incubation mixture	Preincubation pH Preincubation time (min)	...	Activity (% of untreated control activity)												
				Sugar donor ...	UDP-glucuronic acid						UDP-xylose					
					6.8		7.1		7.6		6.8		7.1		7.6	
					30	60	30	60	30	60	30	60	30	60	30	60
					...	30	60	30	60	30	60	30	60	30	60	30
<i>A</i>	(<i>a</i>)		87	76	81	68	74	58	76	59	68	48	57	34		
<i>B</i>	(<i>a</i>)					61		64				49		32		
	(<i>b</i>)					85		88				50		35		
	(<i>c</i>)					86						56		32		
	(<i>d</i>)					86		89				55		32		
<i>C</i>	(<i>a</i>)			59				43		55				39		
	(<i>d</i>)			87				81		61				45		

Table 4. *Apparent K_m values of microsomal bilirubin UDP-glucosyltransferase and bilirubin UDP-xylosyltransferase*

Microsomal preparations were activated with digitonin and assayed as described in the Materials and Methods section. The concentration of one substrate was varied while human serum albumin and the other substrate were kept at their respective standard concentrations. Apparent K_m values were obtained by the method of least squares. K_m values for Mg²⁺ refer to the Mg²⁺-dependent enzyme fractions.

Enzyme	Microsomal preparation no.	Enzyme incubation time (min)	Substrate at varying concn.	Range of concn. used (mM)	Apparent K_m (mM)
Bilirubin UDP-glucosyltransferase	1	10	UDP-glucose	2.6–17.9	3.08
		20		2.6–17.9	3.26
	2	15	Mg ²⁺	0.74–8.1	3.18
	3	10		0.34–5.1	1.8
Bilirubin UDP-xylosyltransferase	4	10		0.17–5.0	1.3
	5	10	UDP-xylose	0.43–2.6	0.72*
		20		0.64–2.6	0.71*
	6	4	Mg ²⁺	0.44–2.6	0.72*
		8		0.44–2.6	0.74*
		30		0.89–7.1	0.34
	8	10		0.19–9.3	0.41

* K_m values for UDP-xylose are representative for the concentration ranges covered by lines no. (1) in Figs. 7–9.

Stabilities of the donor and acceptor substrates

By using incubation mixtures from which bilirubin has been omitted, partial destruction of the UDP-sugars was indicated by release of P_i (Table 2). However, over the incubation periods used as a routine the amounts liberated were small. On the other hand, no loss of bilirubin could be demonstrated when standard microsomal incubation mixtures from which UDP-sugar has been omitted were shaken for 10–120 min at pH 7.1 at 37°C (tests at final bilirubin concn. 0.061 and 0.144 mM). Identical mixtures kept at 0°C served as controls. In similar experiments with replacement of Mg^{2+} (standard system) by Mn^{2+} , Co^{2+} or Ca^{2+} (final concn. 5.77 mM) incubation for 0–60 min produced no changes in the absorption spectrum of the mixtures (absorption maximum in the region of 450 nm), nor in the amounts of total bilirubin (Van Roy *et al.*, 1971);

Cu^{2+} produced an immediate greenish discoloration with a gradual disappearance of the absorption band at 450 nm.

Comparison of the stabilities of bilirubin UDP-xylosyltransferase and bilirubin UDP-glucuronyltransferase activities

By incubating standard microsomal incubation mixtures (without UDP-sugar) at 37°C for various periods of time before initiation of glycosyl transfer (by the addition of the appropriate UDP-sugar), a gradual loss of the enzyme activities was demonstrated (Fig. 3, open symbols). After 10 min of preincubation the activity of UDP-xylosyltransferase was diminished by about 12%, with a smaller loss for the other enzyme. The difference in stability was more pronounced at longer preincubation times.

Fig. 4. Effect of bilirubin concentration on bilirubin UDP-glucosyltransferase

Two microsomal preparations (A and B, indicated by ○, ● and □, ■ respectively) were assayed. The enzyme incubation times were, with preparation A, 6 min (●) and 12 min (○), and with preparation B, 10 min (■) and 20 min (□).

Omission of the albumin–bilirubin substrate mixture during preincubation had little effect on UDP-xylosyltransferase but greatly destabilized the other transferase (Fig. 3, filled-in symbols). Such experiments offer a basis for estimation of enzyme stabilities when assayed under their own appropriate optimum conditions. However, comparison of the inactivation rates is difficult, as during the pretreatments the incubation mixtures were not only at different pH values but also contained different concentrations of microsomal material and digitonin.

To allow a more valid comparison, experiments were done with microsomal preparation and digitonin mixtures fully activated (20h at 4°C) with respect to both enzyme activities (Table 3). In both cases the stabilities decreased from pH 6.6 to 7.6. The presence of human serum albumin in the incubation mixtures stabilized bilirubin UDP-glucuronyltransferase.

Variation of bilirubin and UDP-sugar concentrations

Bilirubin concentration. To a close approximation Michaelis–Menten kinetics were followed with respect to bilirubin (Figs. 4 and 5). If in the double-reciprocal plots the experimental points below $[\text{bilirubin}]^{-1} \text{ (mm)}^{-1}$ equal to 12 are considered to be affected by substrate inhibition, then curve-fitting by the method of least squares gives K_m for bilirubin = 0.22–0.24 mM (with UDP-glucose) and 0.09–0.16 mM (with UDP-xylose).

UDP-sugar concentration. Double-reciprocal plots of activity against the concentration of UDP-glucose were linear (Fig. 6), with no indication of substrate inhibition up to 18 mM-UDP-glucose. Apparent K_m values averaged 3.2 mM (Table 4).

A more complex behaviour was observed with UDP-xylose (Figs. 7 and 8). Apart from a range of inhibitory concentrations, the double-reciprocal

Fig. 5. Effect of bilirubin concentration on bilirubin UDP-xylosyltransferase

Two microsomal preparations were used. Duplicate incubation mixtures were assayed at 4 min (●) and 8 min (○) (preparation no. 1) and at 6 min (■) and 12 min (□) (preparation no. 2).

plots could be approximated by two straight lines (numbered 1 and 2 in the figures). Divergence of the two straight sections increased when the incubation time was prolonged. Deviations at low substrate concentrations (line no. 2 in Figs. 7 and 8) were well outside experimental error. Deviation from constant reaction velocities is apparent at low substrate concentrations (see the insert in Fig. 7).

Lines no. (1) were linear to a high degree (Fig. 9). They thus correspond to hyperbolic parts of the substrate saturation curves (see the inserts in Figs. 7 and 8). Apparent K_m values obtained from these sections are given in Table 4.

Activation by bivalent metal ions

Both transferring enzymes were stimulated by Mg^{2+} (Figs. 10 and 11). On one occasion, when relatively high concentrations were used, 14.3 mM- Mg^{2+} produced 14% inhibition in assays with UDP-glucose and 4.5% inhibition with UDP-xylose. A rather large fraction of microsomal UDP-glucosyltransferase lacked a requirement for bivalent metal ion (Fig. 10, Table 5). In assays with four microsomal preparations this amounted to 45–78% of the activities found in the standard assay system containing Mg^{2+} . Double-reciprocal plots of the corrected activity (total activity – activity found in the absence

Fig. 6. Effect of UDP-glucose concentration on bilirubin UDP-glucosyltransferase

A microsomal preparation was assayed at incubation times 10 min (●) and 20 min (○). The extraction volume was 3 ml.

of added Mg^{2+} against the Mg^{2+} concentration were linear (Fig. 10), whereas curved plots were obtained when the total activities were used. With UDP-xylose as the sugar donor two microsomal preparations showed a nearly absolute requirement for Mg^{2+} (see, e.g., Fig. 11), but six other preparations showed Mg^{2+} -independent fractions amounting to 8–43% of the values found in the standard assay system.

The effects of other bivalent metal ions were tested in parallel on the same microsomal preparations

(Table 6). For comparison enzyme activities were also determined in the absence of added bivalent metal ion. The latter activity was exceeded when one of several bivalent ions was incorporated into the incubation mixtures; Ca^{2+} (final concn. 5.8mM) was slightly less, and Mn^{2+} (final concn. 0.9mM) slightly more, active than Mg^{2+} . Frequently Mn^{2+} can replace Mg^{2+} as an activator of enzymes acting on phosphorylated substrates (Dixon & Webb, 1965). Co^{2+} and Fe^{2+} stimulated glycosyl transfer, whereas

Fig. 7. Effect of UDP-xylose concentration on bilirubin UDP-xylosyltransferase

Duplicate incubation mixtures containing microsomal material were assayed at incubation times 10min (●) and 20min (○). The extraction volume was 3ml. Lines no. (1) and no. (2) refer to concentration ranges where mixtures of mono- and di-xyloside (1) or monoxyloside only (2) are synthesized.

ig. 8. Variation of the activity of bilirubin UDP-xylosyltransferase with the concentration of UDP-xylose. A microsomal preparation was assayed. The incubation time was 15min; the extraction volume was 3ml. The significance of the straight lines no. (1) and no. (2) is outlined in the legend to Fig. 7.

Pb^{2+} was inhibitory. In all cases mentioned sugar transfer was demonstrated unequivocally by t.l.c. of final azopigment extracts.

Assays

Assays with free sugars and with UDP-galactose. With microsomal incubation systems (digitonin-activated and untreated preparations tested) replace-

ment of UDP-sugar by D-glucose, D-xylose or D-glucuronic acid (final concn. 3mM) did not cause measurable formation of conjugated bilirubin (incubation time 15min) compared with incubation without added sugar. In confirmation, no conjugated azopigments could be detected by t.l.c. when portions (1.5ml) of the extracts were analysed. Similarly no conjugation occurred when UDP-galactose (final

Fig. 9. Effect of relatively high concentrations of UDP-xylose on bilirubin UDP-xylosyltransferase

Two microsomal preparations (indicated by ○, ● and □, ■ respectively) were used. Only concentration ranges corresponding to the hyperbolic parts of the saturation curves (cf. line no. 1 in Figs. 7 and 8) were explored. Duplicate incubation mixtures were assayed at 4 min (●) and 8 min (○), and at 10 min (■) and 20 min (□).

concn. 2.54 mM) replaced the standard UDP-sugars in incubation systems containing cell extract (incubation time 16 min) and Mg^{2+} (standard system), Mn^{2+} , Co^{2+} or Zn^{2+} as the activating ions (final concn. for each 5.8 mM).

Assays with liver homogenates from Gunn rats. No significant conjugate formation occurred when either digitonin-activated or untreated liver homogenate was incubated with UDP-xylose, UDP-glucose or UDP-glucuronic acid (Table 7). T.l.c. of 1.5 ml portions of the final organic extracts confirmed this conclusion.

Discussion

The enzyme activities described in the present work have been denoted bilirubin UDP-glucosyltransferase and bilirubin UDP-xylosyltransferase on the basis of two considerations. (1) No conjugate formation occurred with free sugars. (2) The reaction products are monoglycosidic structures with the β -D-configuration at C-1 (Fevery *et al.*, 1972). In these respects the behaviour is similar to that of enzyme systems catalysing the transfer of sugars from sugar nucleotides to acceptor substances (generally

hydrophobic) with the formation of β -D-mono-glycosides. A UDP-glucosyltransferase with these properties occurs in plants, molluscs and insects (Dutton, 1966; Hassid, 1967). Apart from UDP-glucuronyltransferase (Dutton, 1971) several additional UDP-glycosyltransferases have been demonstrated in mammalian tissue: UDP-glucosyltransferases acting on steroids and oestradiol-17 α 3-glucuronoside (Collins *et al.*, 1970; Williamson *et al.*, 1971), ceramide (Basu *et al.*, 1968) and *p*-nitrophenol (Gessner & Vollmer, 1969), and UDP-*N*-acetylglucosaminyltransferase acting on several steroid 3-glucuronosides (Collins *et al.*, 1968). Galactosyl transfer from UDP-galactose to steroids, catalysed by microsomal preparations from rabbit liver, has also been observed (Williamson *et al.*, 1971). Independently from the present work Wong (1971) demonstrated catalysis of glucosyl transfer from UDP-glucose to bilirubin by rat liver microsomal preparations.

Bilirubin UDP-glucosyltransferase and bilirubin UDP-xylosyltransferase show the same behaviour except for differences in susceptibility to stimulation by Mg^{2+} and by other bivalent metal ions. The available evidence thus favours location of both activities, at least for the Mg^{2+} -stimulated fractions, in the same enzyme molecules. This is not necessarily contradicted by the observed synthesis *in vitro* of bilirubin β -D-monoglucoside, in contrast with the formation of both mono- and di-conjugated bilirubin when UDP-xylose is used as the sugar donor (Fevery *et al.*, 1972). It may be noted that ceramide UDP-glucosyltransferase from chicken brain (Basu *et al.*, 1968) was not stimulated by added bivalent metal ion. The problem raised by the real or apparent duality of Mg^{2+} -dependent and -independent UDP-glycosyltransferases has been briefly discussed elsewhere (Heirwegh *et al.*, 1972).

Because glucose and xylose nucleotides other than the respective UDP-sugars were not available the nucleotide requirements of the enzymes could not be established completely. However, according to Wong (1971), ADP-, CDP- and GDP-glucose are inactive as glucosyl donors when bilirubin is the acceptor substrate. Also, with oestradiol-17 α or oestradiol-17 α 3-glucuronoside (Collins *et al.*, 1970) or with ceramide (Basu *et al.*, 1968) as the glucose acceptors, no transfer of sugar occurred with ADP-, CDP-, GDP- or TDP-glucose.

Michaelis-Menten kinetics were not followed with respect to UDP-xylose (Figs. 7–9). The presence of two approximately straight sections in the double-reciprocal plots (Figs. 7 and 8), and the increased divergence found on prolonged incubation, may be related to the sequential formation of mono- and di-xyloside. Indeed, analysis of the reaction products demonstrated (a) dominant or exclusive formation of monoxyloside at low UDP-xylose concentrations

Fig. 10. Effect of concentration of Mg^{2+} on bilirubin UDP-glucosyltransferase

A microsomal preparation was assayed at an incubation time of 10 min. The zero-concentration sample was obtained by replacing the $MgCl_2$ soln. by 0.37 mM-disodium EDTA, pH 7.1 (final concn.). The double-reciprocal plots were obtained by plotting the reciprocal of the (total activity—activity found in the absence of added Mg^{2+}) against the reciprocal of the Mg^{2+} concentration.

(line no. 2 in Figs. 7 and 8) and of mixtures of both conjugates at higher concentrations of the sugar nucleotide (line no. 1), and (b) increased synthesis of dixyloside as incubation proceeded. The next paper (Fevery *et al.*, 1972) gives a full account of these studies. Interpretation of the apparent K_m values (Table 4) obtained from Fig. 9 and from lines no. (1) in Figs. 7 and 8 must be postponed until the mechanism governing sequential formation of the conjugates is better understood. Michaelis-Menten kinetics were followed with respect to UDP-glucose (Fig. 6). Only bilirubin β -D-monoglucoside is synthesized (Fevery *et al.*, 1972).

In several respects bilirubin UDP-glucuronyl-transferase and the enzymes described in the present paper had similar properties (compare the present results with those of Heirwegh *et al.*, 1972). Apart from the similarities in the reaction products mentioned above, the enzyme activities showed closely

parallel distributions over liver cell fractions (Table 1) and were stimulated by digitonin and by Mg^{2+} and several other bivalent metal ions. In view of the observed UDP-glucuronyltransferase deficiency in the Gunn rat (Schmid, 1966) the complete absence of any transfer of sugar from the respective UDP-sugars to bilirubin (Table 7) is compatible with the presumed identity of the three UDP-glycosyltransferases.

Some observations do not easily fit a 'single-enzyme' hypothesis. The shape and localizations of the activity-pH curves, and the ratios of digitonin-activated enzyme activity to untreated enzyme activity are quite different. Further, only bilirubin UDP-glucuronyltransferase is stabilized by human serum albumin (Fig. 3; Table 3). On the whole no definite conclusion can be drawn as yet. As with many problems related to membrane-bound enzymes, studies on more purified preparations are required.

Fig. 11. Effect of concentration of Mg^{2+} on bilirubin UDP-xylosyltransferase.

A microsomal preparation was assayed at an incubation time of 10min. The zero-concentration sample was prepared by replacing the $MgCl_2$ solution by disodium EDTA, pH7.1 (final concn. 0.37mM).

Table 5. UDP-glucosyltransferase activity in the absence of exogenous bivalent metal ion

Digitonin-activated microsomal material was used. Disodium EDTA was omitted from the microsomal preparation, from the digitonin suspension and from the albumin–bilirubin mixture. Enzyme assays were done by the standard procedure outlined in the Materials and Methods section, except for the following: apart from the standard assay (Mg^{2+} present; activity set at 100%) assays were carried out with replacement of the Mg^{2+} soln. by water, or by disodium EDTA, pH7.1, of increasing concentration.

Addition	Concn. (mM)	Enzyme activity	
		(nmol of bilirubin conjugated/ 10min per g wet wt. of liver)	(% of value with Mg^{2+})
Mg^{2+}	7.6	32.0	100
None		24.7	77.6
EDTA	0.51	24.7	77.6
	5.1	24.8	77.8
	15.6	23.0	71.7

Table 6. *Effects of bivalent cations on bilirubin UDP-glycosyltransferases*

Incubation mixtures containing digitonin-activated microsomal material from rat liver and (a) bivalent cation or (b) no such addition (control sample) were assayed in parallel (incubation time: 10min with UDP-xylose; 15min with UDP-glucose). Disodium EDTA was omitted completely from the mixtures. Further details are given in the Materials and Methods section. Enzyme activities are expressed as percentages of the value found with each preparation at 5.77 mM-Mg²⁺ (final concn. in the incubation mixtures). Values found with UDP-glucuronic acid (average values with the number of preparations tested given in parentheses) are indicated for comparison (from Heirwegh *et al.*, 1972).

		Activity (%)							
Donor sugar nucleotide ...		UDP-glucose		UDP-xylose				UDP-glucuronic acid	
Microsomal preparation no. ...	Concn. of added cation (mM)	1	4	1	2	3	4		
Mg ²⁺	5.77	100	100	100	100	100	100	100	
Ca ²⁺	0.91	90	82	65			70	64 (2)	91 (2)
	5.77	100	93	95				92	
Pb ²⁺	0.91	78		33				37 (1)	65 (1)
	5.77		19	23					
Mn ²⁺	0.91	109	115	107	100		105	110 (3)	102 (2)
	5.77	(139)	(72)	95	96			82	
Fe ²⁺	0.91	87	36	49			44	32 (2)	56 (2)
	5.77		116	56	115			86	
Co ²⁺	0.91	84	69	63		57	60	47 (2)	61 (2)
	5.77		87	82	85			86	
None		75	45	32	39	43	38	17-32	

Table 7. *Conjugating activities of liver homogenates from Gunn rat and Wistar rat*

Digitonin-activated homogenates (100mg wet wt. of liver and 4.8mg of digitonin/test) and untreated homogenates (100mg wet wt. of liver/test) from Gunn rat livers were incubated at 37°C for 30min. Otherwise enzyme incubation conditions were exactly as adopted for the standard procedures (see the Materials and Methods section). Results are expressed as nmol of bilirubin conjugated/10min per g wet wt. of liver. Activities obtained with Wistar rat liver homogenates are given (±S.D.) for comparison (taken from Table 1).

				Activity			
Enzyme source	Animal strain	No. of animal	Sugar donor	...	UDP-glucose	UDP-xylose	UDP-glucuronic acid
Untreated homogenate	Gunn rat	1			0.2	0.7	−0.1
		2			2.0	−1.2	−1.3
		3			1.7	0.9	−2.7
	Wistar rat				83 ± 22	149 ± 36	55 ± 10
Activated homogenate	Gunn rat	1			1.1	−0.2	0.5
		2			−0.3	−0.7	−0.7
		3			2.4	0.8	−0.7
	Wistar rat				213 ± 37	318 ± 38	731 ± 116

Until the present date, no truly soluble, active preparations of UDP-glucuronyltransferase seem to be available (Attwood *et al.*, 1971).

The question may be raised whether or not the enzyme activities described in the present work have

any relevance for processes occurring *in vivo*. In dog bile, glucose and xylose conjugates of bilirubin occur (Fevery *et al.*, 1971). Smaller quantities of similar substances have been detected in human bile, and possibly trace amounts in rat bile. Obviously, the

enzymes described in the present paper could participate in the synthesis of such conjugates.

In animal tissues UDP-glucose is present (Keppler *et al.*, 1970) and has been implied in a number of transfer reactions (Hassid, 1967). Much less is known about the occurrence and possible functions of UDP-xylose in animal tissues. This compound may be involved in processes leading to the synthesis of glycoproteins (Grebner *et al.*, 1966a,b) and may be synthesized from UDP-glucuronic acid by decarboxylation (Bdolah & Feingold, 1965; Silbert & DeLuca, 1967).

In contrast with the rates of conjugation observed *in vitro* with rat liver homogenates (Table 1) the biliary excretion of bilirubin glucoside in dog bile (and presumably also in human and rat bile) was greater than that of the xyloside (Feverly *et al.*, 1971). In contrast, with digitonin-activated and with untreated enzyme preparations from rat liver, transfer rates of xylose were greater than those of glucose at saturating concentrations of the substrates (Table 1). A satisfactory explanation for this apparent contradiction cannot be given as yet.

We are grateful to Dr. J. Vandenbroucke and Dr. J. De Groote for their continued support, to Dr. G. J. Dutton for encouragement and for critical appraisal of the text and to the Fonds voor Wetenschappelijk Geneeskundig Onderzoek of Belgium for financial support. The receipt of a post-doctoral fellowship from the Nationaal Fonds voor Wetenschappelijk Onderzoek of Belgium by J. F. is gratefully acknowledged.

References

- Attwood, D., Graham, A. B. & Wood, G. C. (1971) *Biochem. J.* **123**, 875–882
- Basu, S., Kaufman, B. & Roseman, S. (1968) *J. Biol. Chem.* **243**, 5802–5807
- Bdolah, A. & Feingold, D. S. (1965) *Biochem. Biophys. Res. Commun.* **21**, 543–546
- Bock, R. M., Ling, N.-S., Morell, S. A. & Lipton, S. H. (1956) *Arch. Biochem. Biophys.* **62**, 253–264
- Collins, D. C., Jirku, H. & Layne, D. S. (1968) *J. Biol. Chem.* **203**, 2928–2933
- Collins, D. C., Williamson, D. G. & Layne, D. S. (1970) *J. Biol. Chem.* **245**, 873–876
- Compernelle, F., Van Hees, G. P., Fevery, J. & Heirwegh, K. P. M. (1971) *Biochem. J.* **125**, 811–819
- Daughaday, W. H., Lowry, O. H., Rosebrough, N. J. & Fields, W. S. (1952) *J. Lab. Clin. Med.* **39**, 663–665
- de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R. & Appelmans, F. (1955) *Biochem. J.* **60**, 604–617
- Dixon, M. & Webb, E. C. (1965) *Enzymes*, 2nd. edn., pp. 421–426, Longmans Green and Co., London
- Dutton, G. J. (1966) *Arch. Biochem. Biophys.* **116**, 399–405
- Dutton, G. J. (1971) *Handb. Exp. Pharmacol.* **28**, part 2, 378–400
- Feverly, J., Van Hees, G. P., Leroy, P., Compernelle, F. & Heirwegh, K. P. M. (1971) *Biochem. J.* **125**, 803–819
- Feverly, J., Leroy, P., Van de Vijver, M. & Heirwegh, K. P. M. (1972) *Biochem. J.* **129**, 635–644
- Fiske, C. H. & Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
- Gessner, T. & Vollmer, C. A. (1969) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **28**, 545
- Grebner, E. E., Hall, C. W. & Neufeld, E. P. (1966a) *Arch. Biochem. Biophys.* **116**, 391–398
- Grebner, E. E., Hall, C. W. & Neufeld, E. F. (1966b) *Biochem. Biophys. Res. Commun.* **22**, 672–677
- Hassid, W. Z. (1967) *Metab. Pathways*, 3rd. edn., 1, 354–357
- Heirwegh, K. P. M., Van Hees, G. P., Leroy, P., Van Roy, F. P. & Jansen, F. H. (1970) *Biochem. J.* **120**, 877–890
- Heirwegh, K. P. M., Meuwissen, J. A. T. P. & Fevery, J. (1971) *Biochem. J.* **125**, 28p–29p
- Heirwegh, K. P. M., Van de Vijver, M. & Fevery, J. (1972) *Biochem. J.* **129**, 605–618
- Keppler, D., Rudiger, J. & Decker, K. (1970) *Anal. Biochem.* **38**, 105–114
- Kuenzle, C. C. (1970) *Biochem. J.* **119**, 411–435
- Schmid, R. (1966) in *Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B. & Fredrickson, D. S., eds.), pp. 881–883, McGraw Hill Co., New York, Toronto, London and Sydney
- Silbert, J. E. & DeLuca, S. (1967) *Biochim. Biophys. Acta* **141**, 193–196
- Van Damme, B., Fevery, J. & Heirwegh, K. P. M. (1971) *Experientia* **26**, 27–28
- Van Roy, F. P. & Heirwegh, K. P. M. (1968) *Biochem. J.* **107**, 507–518
- Van Roy, F. P., Meuwissen, J. A. T. P., De Meuter, F. & Heirwegh, K. P. M. (1971) *Clin. Chim. Acta* **31**, 109–118
- Williamson, D. G., Polakova, A. & Layne, D. S. (1971) *Biochem. Biophys. Res. Commun.* **42**, 1057–1062
- Wong, K. P. (1971) *Biochem. J.* **125**, 929–934