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Bilirubin Glucuronyltransferase

SPECIFIC ASSAY AND KINETIC STUDIES

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1. Bilirubin glucuronide was synthesized *in vitro* in a system containing a rat liver microsomal fraction, UDP-glucuronic acid, Mg^{2+} and bilirubin. The enzymic synthesis was accomplished without the addition of a bilirubin carrier. 2. Azobilirubin and azobilirubin glucuronide were separated by t.l.c. and paper chromatography and the measurement of the conjugate provided a specific assay for bilirubin UDP-glucuronyltransferase (EC 2.4.1.17). 3. This diazo compound was labelled when [U-^{14}C]UDP-glucuronic acid was employed in the transglucuronidation reaction. 4. Identity of the glucuronide nature of the product was further confirmed by hydrolysis with β-glucuronidase prepared from limpets and *Helix pomatia*. In each instance azobilirubin and glucuronic acid were liberated. 5. There was a close correlation between the bilirubin glucuronyltransferase activity as measured by two procedures, colorimetric and radioisotopic. The specific activities so measured were 19nmol of bilirubin 'equivalents' conjugated/h per mg of protein and 16.9–18.4nmol of UDP-glucuronic acid incorporated/h per mg of protein, respectively. On this basis, it was concluded that the major product formed *in vitro* was bilirubin monoglucuronide; this represents about 77% of the total products formed. 6. The K_m values for bilirubin and UDP-glucuronic acid at pH8.2 are 3.3×10^{-4}M and 1.67×10^{-3}M, respectively. 7. The addition of Mg^{2+} at a final concentration of 5mM to the reaction mixture increased the rate of conjugation by 5.6-fold in the microsomal preparation that had been subjected to overnight dialysis against 10mM-EDTA (disodium salt). 8. Diethylnitrosamine at a final concentration of 1–20mM has no effect on the glucuronidation of bilirubin *in vitro*.

The technical difficulties encountered in the transglucuronidation reaction with bilirubin as substrate are well known (Boerth, Blatt & Spratt, 1965; Dutton, 1966). In addition to this technical problem, there is the question of the validity of the direct van den Bergh reaction as a measurement of bilirubin glucuronide alone; this casts doubt on results obtained in previous studies *in vitro*. Hitherto, two attempts have been made to overcome this dependence on the usual diazotization reaction: first, by using [^{35}S]sulphanilic acid (Metge, Owen, Foulk & Hoffman, 1964) and secondly, by using [^{14}C]bilirubin (Menken, Barrett & Berlin, 1966). Both procedures have not been extensively used. The assay developed by Van Roy & Heirwegh (1968) offers some promise. However, some unconjugated bilirubin was also extracted with the azo-pigments. This interference, though negligible, is undesirable. The low solubility of bilirubin is yet another problem in the kinetic studies of bilirubin glucuronyltransferase. When an aqueous solution of bilirubin was used as a substrate, enzyme activity was often too low to be measurable (Lathe & Walker, 1958). Consequently, for the study of UDP-glucuronyltransferase *in vitro*, bovine serum albumin (Grodsky & Carbone, 1957) or human serum (Lathe & Walker, 1958) was employed as bilirubin carrier. This obligatory inclusion of a substrate carrier often complicated the interpretation of kinetic results, as the effective concentration of bilirubin in a mixture containing bilirubin and albumin or bilirubin and serum was unknown.

In this study, bilirubin glucuronide was shown to be synthesized in a system *in vitro* without the addition of a bilirubin carrier protein. Normal kinetics were obtained and these were comparable with those established by other workers. Chromatographic analysis of the azo-pigments present in the reacted mixture shows that with the excess of bilirubin normally used, it is difficult to eliminate its interference in the direct reaction measurement

(Lathe & Ruthven, 1958). As a corollary, the separation of the conjugated product formed becomes a prerequisite in any assay procedure of bilirubin glucuronyltransferase that is dependent on the diazotization reaction. This was accomplished in this study by t.l.c. and paper chromatography.

MATERIALS AND METHODS

Chemicals. The following were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.): bilirubin; UDP-glucuronic acid (NH_4^+ salt; 98% purity); β-glucuronidase (type H-1) from *Helix pomatia*, and (type L-1) from limpets, containing 650000 and 750000 Fishman units/g, respectively (Talalay, Fishman & Huggins, 1946). [U-^{14}C]-UDP-glucuronic acid, labelled in the glucuronic acid moiety, was from The Radiochemical Centre, Amersham, Bucks., U.K. (50 μCi/ml; specific radioactivity, 302 mCi/mmol); diethylnitrosamine from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; cellulose powder MN-300 from Macherey, Nagel and Co., Duren, Germany and silica gel G from E. Merck A.-G., Darmstadt, Germany.

Preparation of enzyme. Sprague–Dawley rats (200–250 g) were used as a source of enzyme. The animals were killed by decapitation and livers from three or four animals were pooled in the preparation of the microsomal fraction as described by Wong & Sourkes (1967). The pellets were suspended in cold 0.15 M-KCl so that 1 ml of this suspension corresponded to approx. 1 g fresh wt. of liver and contained 10–20 mg of protein. This microsomal preparation was then dialysed overnight at 4°C against 10 mM-EDTA (disodium salt). The dialysis residues, stored frozen in small fractions, retained their activity for at least 1 month. No activation of the enzyme was evident during this period (cf. Lueders & Kuff, 1967). Enzyme activity was also determined in the homogenate (25%, w/v) and in the supernatant and residue obtained after centrifugation at 15000 *g* for 30 min.

Reaction conditions. The reaction mixture for the assay of bilirubin glucuronyltransferase contained the following (final concentrations): bilirubin (0.17 mM); UDP-glucuronic acid (2.97 mM); tris–HCl buffer, pH 8.2 (80 mM); $MgCl_2$, $6H_2O$ (5 mM) and the microsomal fraction containing 1.5–2 mg of protein. The reaction was started by the addition of a freshly prepared solution of bilirubin. The tubes were then tightly stoppered and incubated at 37°C in the Dubnoff shaker bath. At various time-intervals, the reaction was terminated by the addition of 0.1 ml of a concentrated diazo reagent prepared by the procedure of Weber & Schalm (1962).

Extraction of the azo-pigments. Diazotization was allowed to proceed for 10 min at 37°C before the azo-pigments were extracted successively with 1 ml followed with two 0.5 ml portions of butanol. The tubes were thoroughly shaken on the cyclo-mixer and centrifuged for 3 min with a MSE Minor centrifuge at 2000 rev./min. The pooled extract was then washed with half its volume of water.

Analysis of bilirubin glucuronide. (*a*) Qualitative. For qualitative analysis, 200–250 μl of the butanol extract was removed and evaporated to dryness under a stream of N_2. The residue was redissolved in 50 μl of methanol. A portion (10–20 μl) of this was then applied to a microscope slide (25 mm × 75 mm) coated with silica gel G. Development of these plates in histology jars in the solvent system butan-1-ol–acetic acid–water (4:1:4, by vol., upper phase) took only 30 min. The intensity of the azobilirubin glucuronide, as detected on the t.l.c. plate, represented the degree of transglucuronidation. Preferably, this comparison and assessment should be made immediately after the development of the chromatograms, as their prolonged exposure to light inevitably resulted in the fading of the spots. Plates kept in a specimen box retain the coloration of the diazo bands for a longer period of time.

(*b*) Semi-quantitative analysis by densitometry. The t.l.c. plates developed as described above can be readily scanned on the Gelscan (Gelman Instrument Co., Ann Arbor, Mich., U.S.A.) by using a slit width of either 3 mm or 10 mm and slit length of 1 mm. The area of the azobilirubin glucuronide peak produced is a measure of the bilirubin glucuronide formed in the transglucuronidation reaction. Attempts to develop this into a quantitative procedure have not been successful; this is discussed below.

(*c*) Quantitative. For the quantitative measurement of the azobilirubin glucuronide, two procedures have been developed. (i) Colorimetric. One-half to two-thirds of the pooled 2 ml butanol extract was evaporated to dryness. The residue, dissolved in methanol, was quantitatively applied on cellulose-coated plates (20 cm × 20 cm) in the form of a 1.5 cm band. The same solvent system produced a discrete azobilirubin glucuronide spot which can be readily eluted with 1 ml of a 0.2 M-sodium acetate buffer, pH 4, which was saturated with butanol. This solvent system is, however, unsuitable for azobilirubin, which forms a diffuse spot on cellulose plates. The tubes were kept in the dark, a procedure that did not affect the intensity of the azo-pigments. After centrifugation, the eluates were measured at 535 nm on the Beckman DB-G spectrophotometer in microcuvettes of 1 cm light-path.

(ii) Radioisotopic. The conditions of the assay were the same except that 5 μl or 10 μl of [U-^{14}C]UDP-glucuronic acid was included in addition to the unlabelled nucleotide. The residue from 1 ml of the butanol extract was quantitatively applied on Whatman no. 1 paper. A 10 μl sample of the aqueous layer and 20 μl of the washings were also subjected to chromatography, the solvent system being the same as that used in t.l.c. Development took 16 h. Strips (½ in) of the chromatograms, taken from ½ in before the origin to the solvent front, were suspended in vials containing 0.025% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 0.4% (w/v) 2,5-diphenyloxazole in toluene. The radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer.

Hydrolysis by β-glucuronidase. The butanol extracts of treated mixtures which contained high concentrations of the azobilirubin glucuronide were pooled. The glucuronide was separated and isolated by t.l.c. It was subsequently subjected to hydrolysis by β-glucuronidase (type H-1 from *H. pomatia*, containing 1300 Fishman units, or type L-1 from limpets, containing 1500 Fishman units). Incubation was carried out overnight at 37°C in 0.2 M-sodium acetate buffer at pH 5 or 4, respectively. The azo-pigments were re-extracted with butanol and identified by t.l.c. Glucuronic acid in the aqueous phase was

separated on t.l.c. plates coated with silica gel G layers prepared with 0.1M-boric acid and developed in benzene–acetic acid–methanol (1:1:3, by vol.); for its detection, the naphtharesorcinol reagent (Smith, 1960) was employed.

Bilirubin standard. The stock solution of bilirubin (2.5mg/5ml of 0.02M-NaOH) was diluted fivefold with methanol. To 200μl of this solution was added another 0.5ml of methanol followed by 0.1ml of the concentrated diazo reagent. Coupling was allowed to proceed at room temperature for 10min. The product was evaporated to dryness and the residue taken up in 0.2ml of methanol. Portions (10–50μl) of this solution containing 1–5μg of bilirubin were subjected to t.l.c. on silica gel-coated plates (20cm × 20cm). Procedures for the elution and measurement of the extinction of the eluate were carried out as described above.

Protein determination. Protein in the microsomal fraction was measured by the procedure of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Thin-layer chromatography. Bilirubin was glucuronidated in the presence of UDP-glucuronic acid, Mg^{2+} and rat liver microsomal fraction. After diazotization, the treated mixture showed two diazo-positive spots with R_F values of 0.61 and 0.30 on silica gel plates, developed in butan-1-ol–acetic acid–water (4:1:4, by vol.). When UDP-glucuronic acid was omitted from the reaction mixture, only azobilirubin (R_F 0.61) was produced (Fig. 1). The R_F values of these two azo-pigments in various solvent systems are shown in Table 1.

Paper chromatography. On Whatman no. 1 paper, two diazo pigments were also demonstrated; their R_F values are 0.57 and 0.80. These corresponded to those prepared from pig bile by the procedure of Schmid (1957). A profile of each of the radiochromatograms is shown in Fig. 2. Two major labelled peaks were shown in the chromatographed butanol extract. Peak I was diazo-positive and was identified as azobilirubin monoglucuronide. Peak II was tentatively identified as azobilirubin diglucuronide. It did not give the diazo reaction, presumably because of the infinitesimal amount present. There was a proportionate increase in these two radioactive peaks with increasing amount of the labelled nucleotide added to the reaction mixture (Figs. 2*a* and 2*b*). A minor peak, III (R_F 0.21) was possibly that of glucuronic acid 1-phosphate, produced by the pyrophosphorolytic split of the nucleotide (Wong & Lau, 1970). The position of azobilirubin was also indicated on the chromatograms (Fig. 2).

Densitometric analysis. Recordings of the chromatographed extracts of the mixture after reaction showed two symmetrical peaks, corresponding to azobilirubin and azobilirubin glucuronide. The determination of the glucuronide by densitometry could not be accepted with confidence because it was difficult to attain a consistently low background reading.

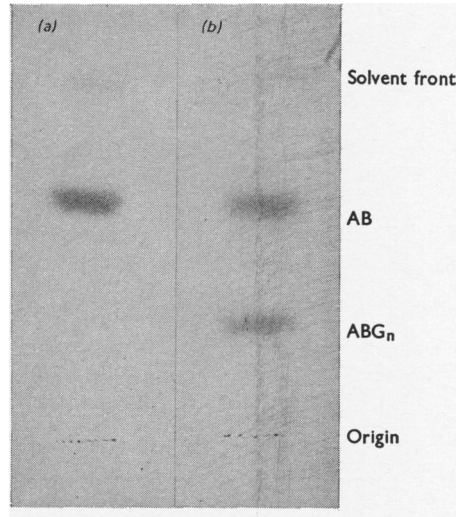

Fig. 1. Thin-layer chromatograms of the butanol extracts of the mixture after reaction on silica gel G-coated plates (25mm × 75mm), developed in butan-1-ol–acetic acid–water (4:1:4, by vol.). In the control (*a*) where UDP-glucuronic acid was omitted, only azobilirubin (AB) was demonstrated. ABG_n represents azobilirubin glucuronide synthesized in the reaction mixture (*b*) which contained 0.5mg of UDP-glucuronic acid.

Table 1. R_F *values of azobilirubin and azobilirubin glucuronide on paper and thin-layer chromatograms*

Experimental details are given in the text. For t.l.c., microscope slides (25mm × 75mm) were used. The solvent systems used were: I, butan-1-ol–acetic acid–water (4:1:4, by vol.); II, methyl ethyl ketone–propan-1-ol–water (15:5:6, by vol.); III, butan-1-ol saturated with 0.2M-sodium acetate buffer, pH4; IV, 0.2M-sodium acetate buffer, pH4, saturated with butan-1-ol.

Solvent systems	Adsorbents	R_F values: Azobilirubin	R_F values: Azobilirubin glucuronide
I	Whatman no. 1	0.80	0.57
I	Silica gel G	0.61	0.30
I	Cellulose	0.67*	0.56
II	Silica gel G	0.56	0.47
II	Cellulose	0.83*	0.61*
III	Silica gel G	0.42	0.06
III	Cellulose	0.70*	0.30*
IV	Silica gel G	0.33*	0.33*
IV	Cellulose	0.17	0.40

* Indicates diffuse spots.

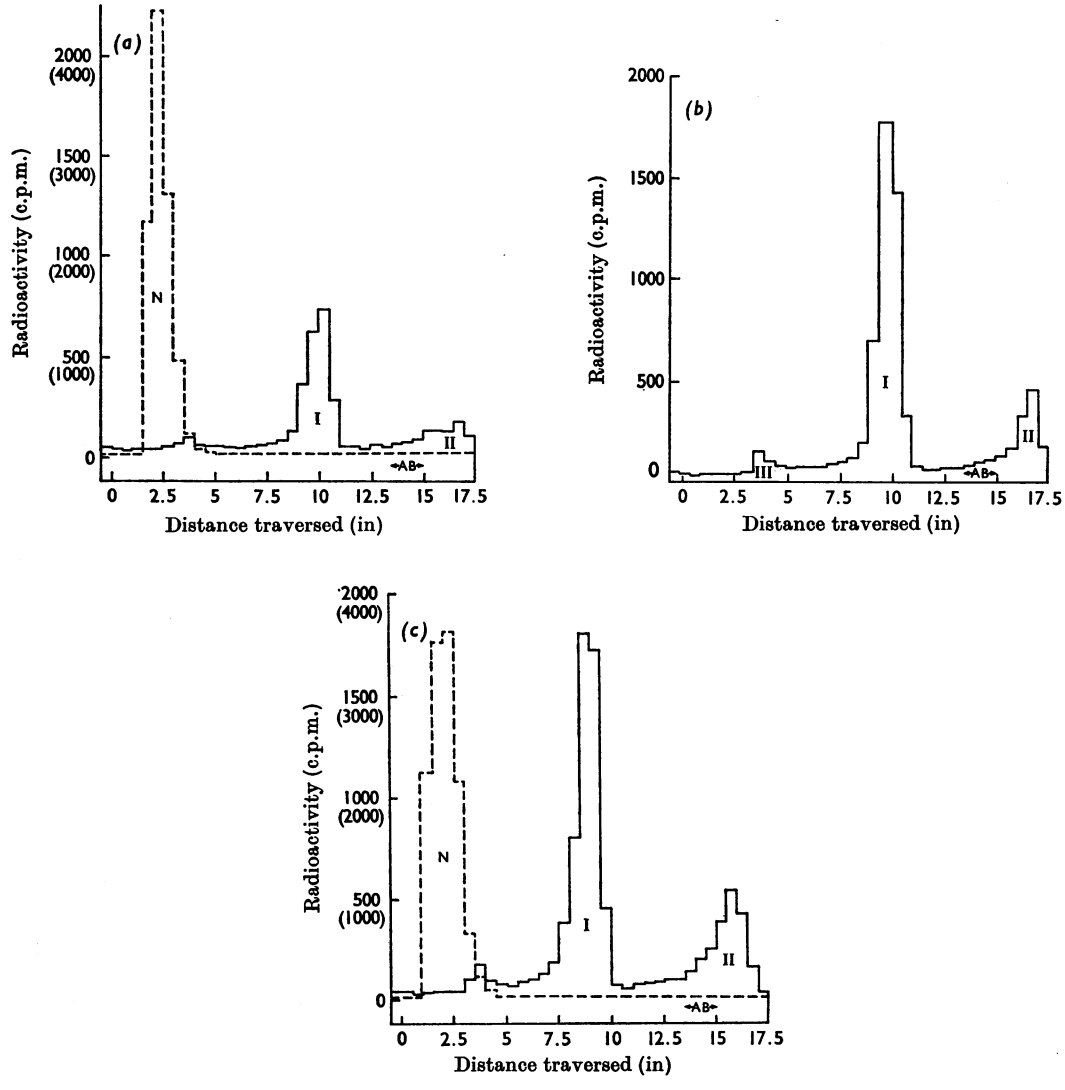

Fig. 2. Paper chromatograms of the butanol (——) and aqueous (----) fractions of the mixture after reaction, containing 5 μl and 10 μl of the labelled UDP-glucuronic acid in addition to the unlabelled nucleotide (*a* and *b* respectively) and only 5 μl of labelled UDP-glucuronic acid (*c*). The ordinate shows the radioactivity (c.p.m.) per fraction of the chromatograms; the values in parentheses represent the radioactivity of the corresponding aqueous fractions. The washings of the pooled butanol extracts did not contain any radioactivity. The abscissa shows the distance (in) traversed in 17 h by radioactive compounds during descending chromatography in butan-1-ol–acetic acid–water (4:1:4, by vol.). Peak N corresponded to the nucleotide UDP-glucuronic acid, which was present only in the aqueous fractions. Peak I was diazo-positive and was identified as azobilirubin monoglucuronide. Peak II did not give the diazo reaction and was tentatively identified as azobilirubin diglucuronide. Peak III has R_F 0.2 and is presumably glucuronic acid 1-phosphate. Azobilirubin occupied a position marked AB on each of the chromatograms.

Standard curve of bilirubin. The extinction of the eluted azobilirubin measured at 535 nm bears a direct relationship to the concentration of bilirubin. Azobilirubin glucuronide produced in the trans-glucuronidation reaction was determined with reference to this standard. Consequently, results are expressed as bilirubin 'equivalents'.

Kinetic results. Kinetic studies of the formation of

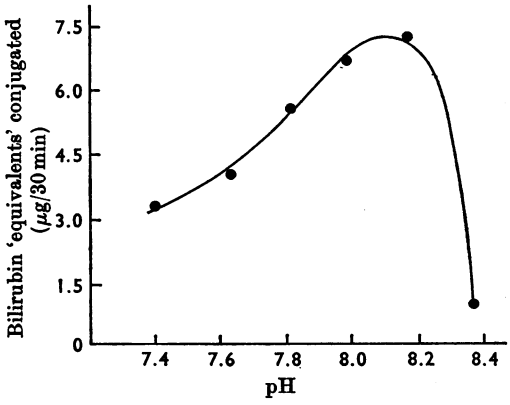

Fig. 3. Effect of pH on bilirubin glucuronyltransferase.

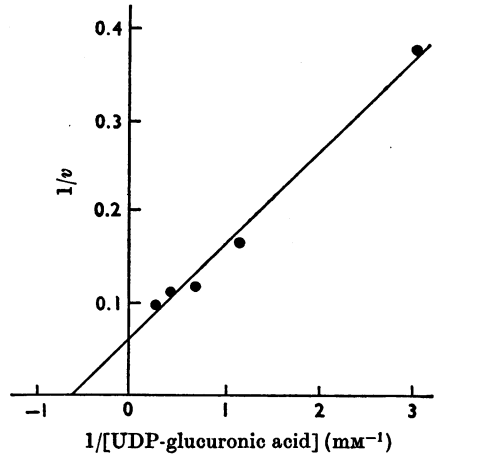

Fig. 4. Double-reciprocal plot of velocity (μg of bilirubin 'equivalents' conjugated/h per mg of protein) against concentrations of UDP-glucuronic acid.

bilirubin glucuronide were carried out by using male rat liver microsomal fractions that had been dialysed against 10mM-EDTA (disodium salt).

(*a*) Effect of pH. The pH–activity curve of the transglucuronidation is shown in Fig. 3. The optimum pH is 8.2.

(*b*) Effect of incubation time. The formation of bilirubin glucuronide increased with time of incubation. No further reaction occurred beyond 30min of incubation.

(*c*) Effect of enzyme concentration. The velocity of the reaction was directly proportional to the amount of enzyme added, up to 1mg of microsomal protein. In this set of experiments, 0.1ml of the enzyme solution contained 1.8mg of protein. To those tubes that contained less than this amount of enzyme protein, a volume of the enzyme solution was added after the diazotization to make up the difference. This was necessary to ensure a distinct partition between the butanol and aqueous phases and to account for any differential adsorption of the azo-pigments on to the freshly precipitated protein.

(*d*) Effect of UDP-glucuronic acid. When the final concentration of bilirubin in the reaction was 0.17mM, conjugation of bilirubin could be demonstrated when UDP-glucuronic acid was present between 0.3 and 3mM. In most experiments, a final concentration of the nucleotide of 1.5–3mM was used. The K_m for UDP-glucuronic acid obtained by the Lineweaver–Burk plot was 1.67×10^{-3}M (Fig. 4).

(*e*) Effect of bilirubin concentration. An alkaline solution of bilirubin was used without the addition of a substrate carrier. From the Lineweaver–Burk plot, the K_m obtained for bilirubin was 3.3×10^{-4}M. Under the conditions of assay, the saturating concentration of bilirubin was 0.09mM.

Effect of Mg^{2+}. The specific activity of bilirubin glucuronyltransferase was increased 5.6-fold by the addition of 2.5μmol of Mg^{2+} (final concentration, 5mM) to the microsomal preparation that had previously been dialysed against 10mM-EDTA (Table 2). The optimum effect was shown at 4mM-Mg^{2+}. At 75mM and above, Mg^{2+} is inhibitory (Fig. 5). This effect of Mg^{2+} with an activity maximum was also demonstrated in rat liver homogenate by Frei (1970). Microsomal preparations dialysed against water or iso-osmotic potassium chloride had lower activity, and the addition of Mg^{2+} ions had negligible action on these preparations.

Hydrolysis by β-glucuronidase. The azobilirubin glucuronide formed enzymically was completely hydrolysed by β-glucuronidase of *H. pomatia* and limpets. In each instance, azobilirubin and glucuronic acid were regenerated (shown by t.l.c. on silica gel G).

Effect of diethylnitrosamine. The carcinogen diethylnitrosamine has no effect on the bilirubin conjugation system when added in a final concentration of 1–20mM. In contrast, there was a 3.5-fold activation of the conjugation of harmol ($C_{12}H_{10}N_2O$, the dehydro-congener of harmalol, 3,4-dihydro-1-methyl-9*H*-pyrido[3,4-*b*]indol-7-ol) with glucuronic acid at 9mM-diethylnitrosamine (K. P. Wong, unpublished work), measured by the procedure of Wong (1969).

Measurement of bilirubin glucuronyltransferase. The activity of various fractions of bilirubin glucuronyltransferase prepared from male and female rat livers are shown in Table 3. Except in the case of the homogenate, enzymic activity is higher in

Table 2. *Specific activity of bilirubin UDP-glucuronyltransferase of liver microsomal preparations of male and female rats*

Experimental details are given in the text. Assays were for 30 min.

Dialysis medium	Specific activity (μg of bilirubin 'equivalents' conjugated/h per mg of protein)	
	Male rat	Female rat
Water	6.9	7.2
KCl (0.15 M)	6.4	6.6
EDTA (1 mM)	7.7	9.1
EDTA (10 mM)	8.2	11.5*

* This enzyme preparation has an activity of 2.04 and 0.7 μg of bilirubin 'equivalents'/h per mg of protein when Mg^{2+} and UDP-glucuronic acid, respectively, were omitted from the reaction mixture.

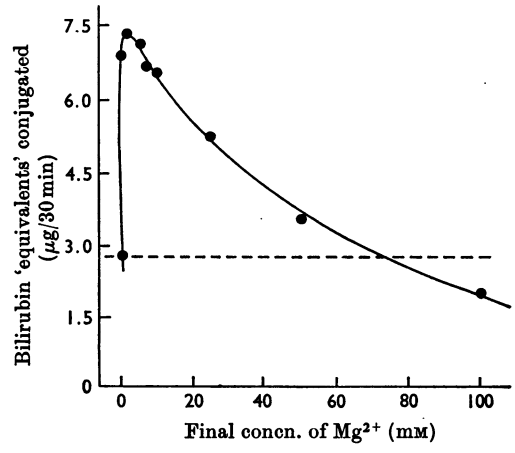

Fig. 5. Effect of Mg^{2+} on bilirubin glucuronyltransferase of rat liver microsomal fraction subjected to dialysis against 10 mM-EDTA (disodium salt). The broken line represents the enzyme activity in the absence of Mg^{2+}.

all the fractions prepared from female rat liver. The specific activities of glucuronyltransferase of the microsomal preparations subjected to different media of dialysis are shown in Table 2. When measured by the colorimetric procedure, the specific activity of female rat liver microsomes was 19 nmol of bilirubin 'equivalents' conjugated/h per mg of protein. The corresponding value obtained by the radioisotopic procedure was 16.9 and 18.4 nmol of UDP-glucuronic acid incorporated/h per mg of protein, respectively, in the two experiments when 5 μl and 10 μl of labelled UDP-glucuronic acid were added. The efficiency of counting of radioactivity was 60%.

DISCUSSION

There are two outstanding limitations in the kinetic study of the formation of bilirubin glucuronide *in vitro*: (i) the dependence on the direct van den Bergh reaction, which is neither qualitative nor quantitative for studies *in vitro*: (ii) the addition of a bilirubin carrier protein to facilitate transglucuronidation makes it difficult to assess the effective concentration of bilirubin in the system. It is well established that the 5 min direct van den Bergh reaction is a measure of solubility and not conjugation. Consequently, a specific determination of bilirubin glucuronidation necessitates the separation of the two azo-pigments. This has been achieved by reverse-phase column chromatography (Cole, Lathe & Billing, 1954) but the procedure is not applicable to studies *in vitro* as the amount of bilirubin glucuronide formed is usually small. The partition procedure of Weber & Schalm (1962) lacks good separation (Boerth *et al.* 1965), whereas that using column chromatography on Chromosorb-G by Lage & Spratt (1968) was time-consuming and more than 4% of the aglycone was reported to appear as a contaminant in the conjugate fraction. The assay procedure of Van Roy & Heirwegh (1968), which depends on the selective coupling of the glucuronide with ethyl anthranilate, provided a more accurate determination of the conjugated bilirubin formed. However, some unconjugated bilirubin was also extracted with the azo-pigments by the solvent mixture used (Black, Billing & Heirwegh, 1970).

The t.l.c. system developed in the present paper provided a specific qualitative and quantitative analysis of the azobilirubin glucuronide synthesized in the reaction. The detection of this azo-pigment on the t.l.c. plate offers a rapid estimation of the bilirubin glucuronyltransferase activity. This method is particularly useful in preliminary work. Semi-quantitative assay of the glucuronide may be made by scanning these chromatograms on the Gelscan. However, inconsistency in the background, attributable to impurities present in the solvent and the unevenness of the silica-gel coating, is an inherent problem in this densitometric measurement. A more practical but less sensitive approach was to elute the azobilirubin glucuronide, followed by spectrometric analysis. Readings so obtained from duplicate reaction tubes are usually consistent, e.g. the specific activity of bilirubin glucuronyltransferase measured in three sets of experiments varies between 11.1 and 11.6 μg of bilirubin 'equivalents' conjugated/h per mg of protein. These results are compared with those established

Table 3. *Activity of bilirubin glucuronyltransferase of various fractions prepared from male and female rat livers*

Experimental details are given in the text. Assays were for 30 min.

Enzyme preparation	Dialysis medium	Specific activity (μg of bilirubin 'equivalents' conjugated/h per mg of protein) Male rat	Female rat
25% (w/v) Homogenate	—	0.95	0.95
	0.15 M-KCl	0.55	0.61
	10 mM-EDTA	1.58	1.42
15000*g* Supernatant	0.15 M-KCl	2.0	3.45
	10 mM-EDTA	2.68	3.60
Mitochondrial fraction	10 mM-EDTA	1.98	2.34
Microsomal fraction	10 mM-EDTA	8.2	11.5

Table 4. *Activity of bilirubin glucuronyltransferase of rat liver*

Enzyme preparations were: H, homogenate; S, slices; M, microsomal. Enzyme activities are expressed as: *, μg of bilirubin conjugated/h per g of liver; †, μg of bilirubin conjugated/h per mg of protein; ‡, μg of bilirubin conjugated/h per mg of N.

Strain and sex of rats	Enzyme preparation	pH	Times of incubation (min)	Mg^{2+} (mM)	Enzyme activity	Reference
—	H	7.4	30	8.8	152–294*	Grodsky & Carbone (1957).
	H	7.4	45	8.8	130*	Grodsky & Carbone (1957).
Wistar	H	7.4	45	8.8	84–96*	Grodsky, Carbone & Franska (1958).
Wistar	H	6.4	20	12	439*	Lathe & Walker (1958).
Wistar	S	6.4	120	12	57*	Lathe & Walker (1958).
—	H	7.5	90	—	193*	Talafant & Tovarek (1959).
Sprague–Dawley	H	7.4	30	8.8	109–143*	Metge *et al.* (1964).
Wistar (male)	H	7.4	15	10	320*	Bevan, Holton & Lathe (1965).
Wistar (male)	S	7.4	15	10	71*	Bevan *et al.* (1965).
Wistar (male)	H	7.9	30	62.5	150–290*	Menken *et al.* (1966).
Wistar (female)	H	7.9	30	62.5	80–170*	Menken *et al.* (1966).
Wistar	S	6.4	120	12	57*	Flint, Lathe, Ricketts & Silman (1964).
Sprague–Dawley	M	8.0	20	30	1332*	Halac & Reff (1967).
Wistar (male)	M	8.2	30–60	10	168–179*	Van Roy & Heirwegh (1968).
					11.7†	Van Roy & Heirwegh (1968).
	M	8.2	10	6.4	343.2*	Van Roy & Heirwegh (1968).
Wistar	M	7.4	—	8.1	121.6‡	Strebel & Odell (1969).
					20†	
Sprague–Dawley	M	7.4	—	8.1	75.4‡	Strebel & Odell (1969).
					12†	
Wistar (male)	S	6.4	120	12	62*	Adlard, Lester & Lathe (1969).
Sprague–Dawley	H	7.4	40	10	2.19†	Mowat & Arias (1970*a*).
Wistar	H	7.4	40	10	2.63*	Mowat & Arias (1970*a*).
Sprague–Dawley	H	7.4	30	10	1500*	Black, Billing & Heirwegh (1970).
Wistar	H	7.4	30	10	2294*	Thaler (1970).
Sprague–Dawley (male)	H	8.2	30	5	0.95†	This paper
Sprague–Dawley (female)	H	8.2	30	5	0.95†	This paper
Sprague–Dawley (male	M	8.2	30	5	98.4*	This paper
					8.2†	This paper
Sprague–Dawley (female)	M	8.2	30	5	138.0*	This paper
					11.6†	This paper

by other workers (Table 4). The enzyme activity measured in homogenates and slices of rat liver varies considerably. The value for microsomal activity presented in the present paper is in close agreement with those reported by Van Roy & Heirwegh (1968) and Strebel & Odell (1969) but lower than that given by Halac & Reff (1967) (see Table 4). In the absence of exogenous UDP-glucuronic acid, very little bilirubin glucuronide was produced. This basal value was usually low (approx. 0.7 μg of bilirubin 'equivalents'/h per mg of protein). Incubation in air or nitrogen showed no difference in the rate of glucuronidation. It was noted that microsomal preparations of male and female rat liver that had been dialysed against water or 0.15M-KCl showed comparable activity (Table 2). However, dialysis against 10mM-EDTA resulted in a consistently higher activity in female rat liver microsomal fraction. It could be inferred from this that dialysis against EDTA could have removed an inhibitory factor that was present in higher concentration in female rat liver.

In a 30min assay, 24% of the bilirubin added was glucuronidated. This represents a value of 19nmol of bilirubin 'equivalents' conjugated/h per mg of protein. When additional 5 μl and 10 μl portions of the labelled nucleotide were introduced into the reaction mixture, the radioactivity incorporated into bilirubin glucuronide constituted 1.22 and 1.33% of the total radioactivity, respectively. This corresponded to 16.9 and 18.4nmol of UDP-glucuronic acid incorporated/h per mg of protein. There is thus a close correlation between the colorimetric and radioisotopic measurements. On the basis of the above results, it is conceivable that peak I, representing the major product formed *in vitro*, contained bilirubin monoglucuronide. This agrees with the conclusions of Van Roy & Heirwegh (1968) and Strebel & Odell (1969). The identity of peak II, believed to be that of bilirubin diglucuronide, deserves further study, as the diglucuronide has never been demonstrated *in vitro*. The peak I/peak II radioactivity ratios are 3.5 and 3.1 in the two experiments. Thus, about 77% of the product formed is bilirubin monoglucuronide. It must be emphasized that the adsorption of the azo-pigments on to the precipitated protein was not considered in the calculations of results, although a great predominance of the azo-bilirubin glucuronide is thought to be preferentially adsorbed on the denatured protein (Cole & Lathe, 1953). It is believed that any loss incurred by this differential adsorption was insignificant, as there was little difference between duplicate and triplicate results. A systematic study to test the efficiency of extraction of the azo-pigments from the reacted mixture by butanol indicated that 2ml of butanol was adequate for complete extraction.

The conjugation of bilirubin with glucuronic acid *in vitro* is low in the absence of a bilirubin carrier (Lathe & Walker, 1958). This was believed to be due to the unspecific binding of bilirubin to microsomal material. This could only be overcome by the addition of albumin or serum to the substrate. In the present study, no substrate carrier was added and normal kinetics were obtained. The velocity of the reaction increased with time of incubation and with concentrations of enzyme, bilirubin and UDP-glucuronic acid. The K_m values with respect to bilirubin and UDP-glucuronic acid by Lineweaver–Burk reciprocal plots are compared with those of other workers in Table 5. In each set of results, it was noted that the relative affinity of the enzyme for bilirubin was 4–6 times that of the conjugating agent.

Various lines of evidence for the existence of multiple UDP-glucuronyltransferases have been presented (Dutton, 1966); this accounts for the different effects of Mg^{2+} on different substrates. The results obtained in this study confirmed the Mg^{2+} requirement of bilirubin glucuronyltransferase (Tomlinson & Yaffe, 1966; Van Roy & Heirwegh, 1968). The addition of 2.5 μmol of magnesium chloride to the microsomal preparation that had been dialysed against 10mM-EDTA more than restored the original activity (Table 2). It is evident that in dialysis some factor(s) other than Mg^{2+} ions were removed or destroyed.

An increase in the glucuronyltransferase activity towards *o*-aminophenol was elicited by the addition of 8–16mM-diethylnitrosamine to rat liver microsomal preparation or homogenate (Greenwood & Stevenson, 1965; Stevenson, Greenwood & McEwen, 1968; Winsnes, 1969; Mowat & Arias, 1970*b*). The stimulatory action of this carcinogen was not shown in the present work with bilirubin as a substrate. The different effects of Mg^{2+} ions and diethylnitrosamine on conjugations of bilirubin, harmol and harmalol (Wong & Sourkes, 1968) further supported the hypothesis of the multiplicity of UDP-glucuronyltransferase.

Table 5. K_m *values of bilirubin and UDP-glucuronic acid*

pH	Bilirubin	UDP-glucuronic acid	Reference
8.0	1.2×10^{-4}M	7.0×10^{-4}M	Halac & Reff (1967)
8.2	1.7×10^{-4}M	6.8×10^{-4}M	Van Roy & Heirwegh (1968)
7.4	0.9×10^{-4}M	—	Van Roy & Heirwegh (1968)
7.9	—	4.6×10^{-4}M	Adlard & Lathe (1970)
8.2	3.3×10^{-4}M	16.6×10^{-4}M	This paper

The principal factors that made the study of bilirubin *in vitro* a notoriously difficult problem have been elaborated. This explains the extensive use of non-physiological aglycones like *p*-nitrophenol, *o*-aminophenol and phenolphthalein in studies of transglucuronidation, as their transformations can be easily and accurately measured. However, results obtained in such investigations cannot be extrapolated to glucuronidation of bilirubin with any confidence. This accounts for the continual search for a simple, accurate and specific assay procedure for bilirubin glucuronyltransferase. This is of immediate practical importance in drug-toxicity investigations of conditions involving transglucuronidation of bilirubin. It is hoped that the simple and specific assay procedure presented here would lend impetus to work in this field. In this connexion, the rat appears to be the most suitable animal for such investigative purposes since it possesses the highest bilirubin glucuronyltransferase activity compared with the mouse or guinea pig. Though both β-glucuronidase and UDP-glucuronic acid pyrophosphatase activities are high in the rat liver, their interference could not be significant because the former has negligible action at the alkaline pH used in the reaction (Wong, 1970) and the latter has been shown to be inhibited by EDTA (Wong & Lau, 1970).

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