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STUDIES ON BILIRUBIN UDP-GLUCURONYLTRANSFERASE

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SUMMARY

The capacity of liver fractions to conjugate bilirubin and *p*-nitrophenol with glucuronic acid is increased several fold by dialysis against alkaline EDTA. UDP-glucuronyltransferase (UDP-glucuronate glucuronyltransferase (acceptor-unspecific), EC 2.4.1.17), with activity towards bilirubin and *p*-nitrophenol, has been solubilized by treating microsomes activated with EDTA and deoxycholate.

Kinetic data obtained with activated microsomes, and fractionation and inactivation experiments performed with deoxycholate-solubilized enzyme have yielded evidence in favor of the existence of at least two UDP-glucuronyltransferases.

Microsomal pellets were examined by electron microscopy before EDTA, after EDTA, and after EDTA and deoxycholate. The most apparent change that is concomitant with the activation by EDTA is the transformation of rough membranes into smooth membranes.

INTRODUCTION

The transformation of bilirubin into bilirubin glucuronide is mediated by the enzyme UDP-glucuronyltransferase (UDP-glucuronate glucuronyltransferase (acceptor-unspecific), EC 2.4.1.17), which catalyzes the transfer of glucuronic acid from UDP-GlcUA to the carboxylic group of bilirubin[1]. Phenolic compounds like *p*-nitrophenol and phenolphthalein form ether conjugates with GlcUA and have been used extensively in studies of UDP-glucuronyltransferase. The results obtained with these substrates have sometimes been used to reach conclusions on the conjugation of bilirubin. This kind of extrapolation would be legitimate only if a single enzyme were responsible for the transfer of GlcUA to different acceptors, as proposed by AXELROD[2].

The techniques reported in the literature which utilize liver homogenates to catalyze the formation of bilirubin glucuronide suffer from serious drawbacks. When we attempted to study this reaction following a published procedure[3], the conjugation values we obtained, as well as those in the original publication, were much lower than

Abbreviation: GlcUA, glucuronic acid.

those expected from the bilirubin excretion capacity of the liver[4]. Furthermore, in our experience the preparations obtained did not lend themselves to kinetic studies. BOERTH, BLATT AND SPRATT[5] have recently evaluated available methods for the study of this reaction and have reached conclusions that are similar to ours.

Dialysis of liver fractions against EDTA increases by several fold their capacity to form phenolphthalein glucuronide[6,7]. We now wish to report a similar EDTA-induced activation of the conjugation of bilirubin and *p*-nitrophenol with GlcUA. We have also been able to solubilize the enzyme by the use of deoxycholate. The results obtained are compatible with the existence of a UDP-glucuronyltransferase for bilirubin different from the enzyme that catalyzes the formation of *p*-nitrophenol glucuronide. A brief report of this work in its preliminary stages has been made elsewhere[8].

MATERIALS AND METHODS

All the reagents were obtained from commercial sources. Ammonium sulphate was recrystallized twice before making the final saturated solution, which contained 1.0 ml of conc. NH_4OH and 1 mmole of EDTA per l. The diphenylacetic acid was crystallized once from an alcoholic solution. The livers were obtained for most of the experiments from male Sprague-Dawley rats that weighed between 200 and 400 g.

The livers are homogenized in a Waring blendor in 4 vol. of cold 0.14 M KCl–0.01 M Tris (pH 8.0). The homogenate is centrifuged at 2000 × *g* for 15 min and 0.7 ml of satd. $(NH_4)_2SO_4$ is added per 1.0 ml of the supernatant (0–42% saturation). The precipitate is collected by centrifugation and suspended in the homogenizing buffer; the volume of each fraction is kept at about 2/3 of the volume of the preceding fraction. The $(NH_4)_2SO_4$ fraction is dialyzed for 18 h against 100 vol. of 0.01 M EDTA–0.2 mM mercaptoethanol (pH 9.0) and then is centrifuged at 105 000 × *g* for 45 min. The pellet is homogenized in 0.3% deoxycholate–1 mM EDTA (pH 8.0). and centrifuged as in the last step. The precipitate is discarded and the supernatant is passed in 10-ml aliquots through a Sephadex G-200 column, 20 cm in length and 2.5 cm in diameter, equilibrated with 0.05 M Tris (pH 8.0)–1 mM EDTA. The void volume of such a column is 30 ml; the first 30 ml of the effluent are discarded and the following 15 ml are collected. This fraction contains the soluble UDP-glucuronyltransferase.

The composition of the assaying mixtures for *p*-nitrophenol, phenolphthalein,

TABLE I

COMPOSITION OF ASSAYING MIXTURES

Reagent	*Acceptor substrate*		
	Phenolphthalein (ml)	*p-Nitrophenol (ml)*	*Bilirubin (ml)*
1.0 M Tris (pH 8.0)	0.6	0.6	0.6
0.01 M substrate	0.2	0.2	0.2
1.0 M $MgCl_2$	0.08	0.01	0.32
10% bovine serum albumin	0.6	0.24	0.3
0.2 M mercaptoethanol	0.04	0.04	0.04
Water	0.48	0.91	0.54

and bilirubin are shown in Table I. The assay for phenolphthalein conjugation was done as previously described[7]. The same procedure was slightly modified for the assay of the conjugation of the other two substrates. The incubation tubes contained 50 μl of the assaying mixtures and the enzyme preparations to a final volume of 100 μl for the *p*-nitrophenol assay and 250 μl for bilirubin. The reaction was started by the addition of 0.1 μmole of UDP-GlcUA (5 μl) for the phenolic acceptors and 0.4 μmoles (20 μl) for bilirubin. Incubations were done at 38° for 5–10 min for *p*-nitrophenol and for 15–30 min for bilirubin. The *p*-nitrophenol reaction was stopped by the addition of 2.0 ml of 0.4 M glycine (pH 10.4). The difference in color between a blank that received no UDP-GlcUA and the test that had UDP-GlcUA represented the substrate glucuronide. The wavelengths used were 410 mμ for *p*-nitrophenol and 555 mμ for phenolphthalein and the millimolar absorbance coefficients used were 17.5 and 25.1, respectively.

Bilirubin glucuronide was assayed by the method of WEBER AND SCHALM[9]. When turbidity was present in the aqueous phase, 0.5 ml of chloroform was added to this phase. After mixing by shaking, the chloroform was eliminated by centrifugation; this resulted in samples that were completely transparent. The color developed by the diazo reagent had maximal absorption at 590 mμ. Readings were taken at 590 and 500 mμ and the ΔA_{590}–A_{500} was taken as proportional to the amount of bilirubin glucuronide. The millimolar absorbance coefficient for bilirubin glucuronide under these conditions was found to be 9.3. A tube incubated without UDP-GlcUA served as the blank. All absorbance measurements were made with a Beckman DU-2. One unit of glucuronyltransferase was defined as that effecting the conjugation of 1 mμ-mole of phenolphthalein or *p*-nitrophenol in 10 min at 38°. A similar unit was defined for the formation of bilirubin glucuronide: the conjugation of 1 mμmole of bilirubin in 30 min at 38°.

Chromatography of the products of the reaction was carried out as described by SCHMID[10]. The aliquots for chromatography received at the end of the incubation 1.0 ml of water and 0.3 ml of the concentrated diazo reagent described by WEBER AND SCHALM[9]. After letting the tubes stand for 1 h in the dark, 1.0 ml of saturated ammonium sulphate was added followed by 1.0 ml of *n*-butanol. The tubes were shaken and the butanol layer was separated and evaporated under vacuum. The residue was dissolved in a small amount of 1.0 M acetic acid and was applied to Whatman 3 MM paper for ascending chromatography.

β-Glucuronidase was measured with an assay mixture identical to the one used for *p*-nitrophenol conjugation, except that phenolphthalein glucuronide substituted for *p*-nitrophenol; other conditions were also identical. The blank received the enzyme preparation after the addition of the glycine buffer. The phenolphthalein liberated was estimated as the difference in absorbance at 555 mμ between the blank and the test tube.

Protein determinations were done with the Folin–Ciocalteu reagent[11].

Electron microscopy

The microsomal pellets were examined by electron microscopy before EDTA treatment, after EDTA and after EDTA–deoxycholate treatment. Samples from these pellets were prepared following the technique of PARSONS[12], by dipping a needle into the pellet and then into a 3% phosphotungstic acid solution. The material was picked

up by touching it with a carbon formvar grid and it was immediately examined with a Siemens Elmiskop I microscope.

RESULTS

To study the formation of bilirubin glucuronide we used first the technique described by GRODSKY AND CARBONE[3]. Conjugations obtained ranged from 0 to 60 μg of bilirubin glucuronide per g of liver in 30 min. We then attempted to measure the bilirubin glucuronide synthesized in the course of the incubation by the method of

TABLE II

UDP-GLUCURONYLTRANSFERASE IN LIVER FRACTIONS BEFORE AND AFTER EDTA TREATMENT

Figures between parentheses are the average activations found in 6 experiments of this kind.

Fraction	*Bilirubin conjugation*			*p-Nitrophenol conjugation*		
	Before EDTA (units/ml)	*After EDTA (units/ml)*	*Activation*	*Before EDTA (units/ml)*	*After EDTA (units/ml)*	*Activation*
Liver homogenate	24	182	8 (8)	160	1010	6 (9)
2000 × *g* for 15 min, supernatant	20	190	8 (8)	150	990	7 (9)
0–42% ammonium sulfate fraction	10	240	24 (13)	90	1300	14 (18)

WEBER AND SCHALM. For this we designed an incubation mixture similar to the one described under METHODS. Under these conditions we obtained a maximum conjugation of 100 μg per g of liver in 30 min. The results obtained with these two techniques were difficult to reproduce and kinetic studies were not possible.

Since dialysis against alkaline EDTA produces a marked stimulation of the

TABLE III

UDP-GLUCURONYLTRANSFERASE IN DIFFERENT ANIMALS

Liver homogenates were dialyzed for 20 h against EDTA and then filtered through gauze. Incubation time was 10 min for *p*-nitrophenol and 20 min for bilirubin. The usual amount of protein added to the assaying tubes was 2.5 mg for the *p*-nitrophenol conjugation and 12.5 mg for bilirubin conjugation. The number of animals used for these experiments is indicated by the figures between parentheses. Values are averages.

Source of liver	*Compound conjugated*	
	p-Nitrophenol (μmoles conjugated per g liver per 10 min)	*Bilirubin (μmoles conjugated per g liver per 30 min)*
Rat (20)	5.2	1.14
Rabbit (4)	15.3	0.62
Guinea pig (5)	8.2	0.25

formation of phenolphthalein glucuronide we assayed liver homogenates after such treatment for their capacity to conjugate bilirubin and *p*-nitrophenol with glucuronic acid. We found that the transfer reaction was increased by 5–15 fold over the original values. Table II shows the activity of three different fractions of rat liver towards *p*-nitrophenol and bilirubin before and after activation with EDTA. The large activation found with the ammonium sulphate fraction is due to the simultaneous removal of SO_4^{2-} which inhibits UDP-glucuronyltransferase[7].

The capacities for the conjugating of *p*-nitrophenol and bilirubin of livers from rats, rabbits and guinea-pigs were compared and the results appear in Table III. The animal that had the highest capacity to conjugate bilirubin had the lowest capacity to conjugate *p*-nitrophenol and *vice versa*. One human liver examined conjugated 12 μmoles of *p*-nitrophenol and 0.98 μmole of bilirubin in 30 min per g of liver tissue.

The activation induced by EDTA at pH 9.0 was not immediate. We followed the time course of this activation at three temperatures: 3°, 20° and 37°. The results of one such experiment carried out at 3° are presented in Fig. 1, where the slight dis-

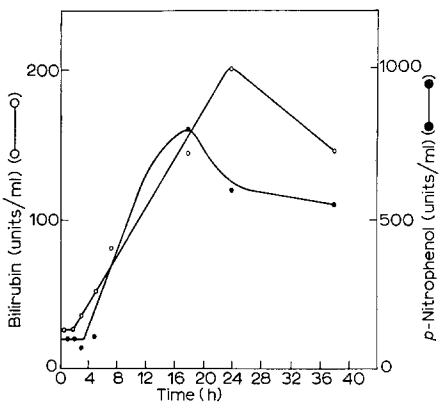


Fig. 1. The increase in UDP-glucuronyltransferase activity of a liver fraction (2000 × *g* for 15 min, supernatant) induced by dialysis against 0.01 M EDTA (pH 9.0)–0.2 mM mercaptoethanol. Enzyme activity was followed with bilirubin (○—○) and *p*-nitrophenol (●—●).

crepancy between the curves for activation of bilirubin and *p*-nitrophenol conjugation is apparent. The activation of the *p*-nitrophenol conjugation at 3° started 2–3 h after the onset of the activation of the reaction that conjugates bilirubin.

The activation by EDTA was accelerated when the temperature at which dialysis was carried out was raised. The maximum bilirubin-conjugating capacity was reached at 3 h, 7 h and 18 h when the dialysis was done at 37°, 20°, and 3°, respectively.

The activity towards *p*-nitrophenol of liver microsomes that had not been activated by dialysis against EDTA was decreased by 16% on the addition of EDTA at a concentration of 5 mM, but when the incubation time was prolonged from 10 min to 30 min a 49% stimulation was found. The reaction with bilirubin catalyzed by similar fractions was not accelerated if the incubation time was 10 min but it was increased 100% by 30 min.

The solubilization of bilirubin-conjugating activity by dialysis against EDTA was between 15 and 22% for rabbit-liver fractions and not greater than 10% for

similar rat-liver fractions. Since rabbit liver has less activity towards bilirubin than rat liver it was evident that this method of solubilization, while practicable for *p*-nitrophenol activity, held no promises for the bilirubin-conjugating enzyme. It was then found that deoxycholate induced the solubilization of UDP-glucuronyltransferase activity in yields sufficient to secure adequate activity for enzymic characterization and fractionation procedures. The removal of deoxycholate is a prerequisite to the assay of UDP-glucuronyltransferase because of its inhibitory action. A 1.2 mM concentration of this compound produced a 40% and 92% inhibition of the *p*-nitrophenol and bilirubin conjugation, respectively. Removal of deoxycholate was accomplished by passing aliquots of the deoxycholate-treated fractions through Sephadex, but it seems likely that removal of deoxycholate was not complete because, when a soluble preparation was made 50% saturated with $(NH_4)_2SO_4$, the protein aggregates formed a film on the top of the liquid column after centrifugation.

When the microsomal fraction homogenized in 0.3% deoxycholate–1 mM EDTA is centrifuged at 105 000 × *g* for 45 min, three layers become easily distinguishable: a top layer where fat is abundant and sometimes in large globules, a middle transparent layer which is the largest in volume, and a pellet. We separated these three fractions and assayed them for capacity to form *p*-nitrophenol and bilirubin glucuronides after passing 2.0-ml aliquots of each fraction through 6.0 ml Sephadex G-200 columns equilibrated with 0.05 M Tris–1 mM EDTA at pH 8.0. Table IV shows the result of an experiment of this kind.

TABLE IV

DISTRIBUTION OF UDP-GLUCURONYLTRANSFERASE ACTIVITY AFTER CENTRIFUGATION

	Bilirubin conjugation (units/ml)	*p-Nitrophenol conjugation (units/ml)*	*Bilirubin (units) / p-Nitrophenol (units)*
Upper layer	45	137	0.33
Middle layer	43	188	0.23
Pellet	44	480	0.09

The ratios between the *p*-nitrophenol and the bilirubin conjugase activities in the three fractions were different. While the glucuronyl transferase activity responsible for the conjugation of bilirubin was uniformly distributed in the three layers, the activity towards *p*-nitrophenol showed a clear gradient from top to bottom, where it was greatest.

The separation of *p*-nitrophenol from bilirubin-conjugating activity was attempted by the use of a Sephadex G-200 column (30 cm in length and 2.5 cm in diameter) equilibrated with the Tris–EDTA buffer. After the measured void volume had passed (about 30 ml), 3.0-ml fractions were collected and assayed for glucuronyl transferase activity with both substrates. The result of an experiment of this kind is shown in Fig. 2. No separation was accomplished but the pattern of elution was different for the two activities. The *p*-nitrophenol conjugase activity appeared clearly divided in two peaks. The bilirubin conjugase activity overlapped both peaks but the maximum activity coincided with the second *p*-nitrophenol peak and a shoulder

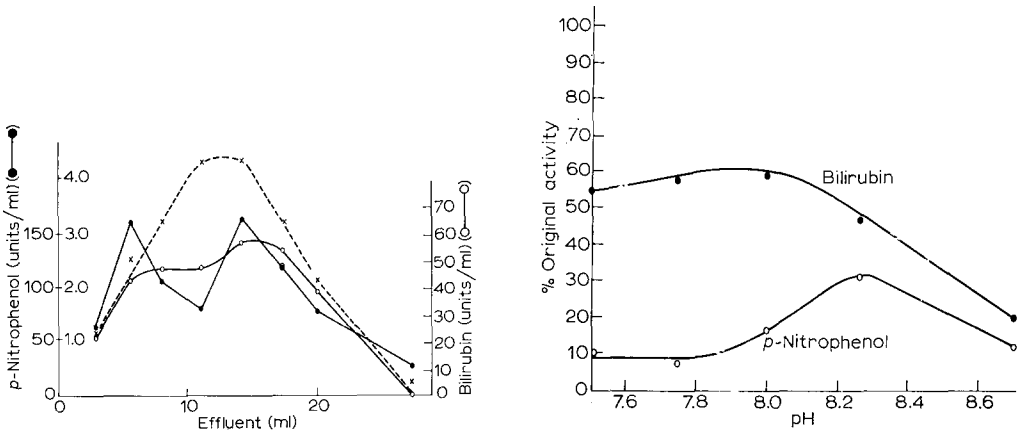


Fig. 2. Elution of UDP-glucuronyltransferase from a Sephadex G-200 column. The column was equilibrated with 0.05 M Tris (pH 8.0)–1 mM EDTA. 10.0 ml of the EDTA–deoxycholate treated microsomal fraction were applied to the top of the column. The activity towards bilirubin (○—○) and *p*-nitrophenol (●—●) and the protein content (×- - -×) was measured in each fraction.

Fig. 3. The effect of pH on the stability of UDP-glucuronyltransferase under heat. Enzyme activity was measured with bilirubin (●—●) and *p*-nitrophenol (○—○).

was present which coincided with the first peak. The protein distribution showed no evidence of such partition.

We studied the stability of both activities in the soluble preparation by heating it at 55° for 10 min at pH values ranging between 7.5 and 8.5. As shown in Fig. 3, the percentage of *p*-nitrophenol activity remaining after heating was very low, and the patterns of inactivation of each of the two activities were different. The optimum pH for the stability of the glucuronyl transferase activity responsible for the formation of bilirubin glucuronide was 0.4–0.5 unit of pH lower than the optimum pH for the

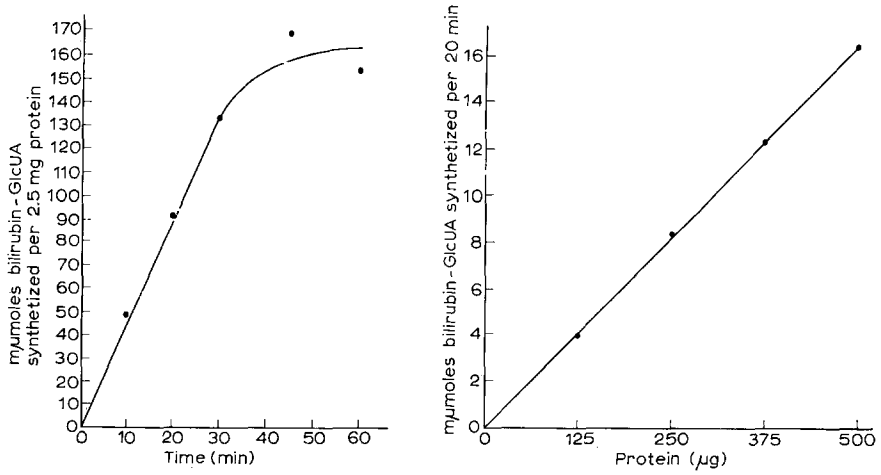


Fig. 4. The effect of protein concentration and incubation time on the conjugation of bilirubin with GlcUA.

p-nitrophenol conjugase activity. This difference in stability afforded a rapid and simple way for obtaining a preparation that had about 60% of the original bilirubin conjugase activity and 10% or less of activity towards *p*-nitrophenol.

Kinetics

The activity of UDP-glucuronyltransferase in EDTA-treated liver fractions was proportional to the time of incubation and to the amount of protein added to the incubation mixture. Fig. 4 shows the results obtained when a soluble preparation was assayed for the formation of bilirubin glucuronide under variable conditions for these two parameters.

The Michaelis constants for different substrates and cofactors were measured utilizing the activated microsomal preparation. The soluble preparation obtained after deoxycholate gave essentially the same values for the bilirubin system.

TABLE V

UDP-GLUCURONYLTRANSFERASE K_m VALUES MEASURED WITH DIFFERENT ACCEPTORS

K_m *measured for*	*Acceptor substrate*		
	Bilirubin	*p-Nitrophenol*	*Phenolphthalein*
Bilirubin	$1.2 \cdot 10^{-4}$	—	—
p-Nitrophenol	—	$5.5 \cdot 10^{-4}$	—
Phenolphthalein	—	—	$6.6 \cdot 10^{-5}$
UDP-GlcUA	$7.0 \cdot 10^{-4}$	$3.0 \cdot 10^{-4}$	$1.8 \cdot 10^{-5}$
$MgCl_2$	$9.0 \cdot 10^{-3}$	$1.8 \cdot 10^{-3}$	Not measured

Bovine serum albumin stimulates the formation of phenolphthalein glucuronide as well as the conjugation of *p*-nitrophenol and bilirubin. The requirements for the different acceptors were found to be quite exacting. The optimum concentration for the bilirubin, *p*-nitrophenol and phenolphthalein reactions were 3.0, 6.0 and 15.0 mg per ml of incubation mixture. Slightly higher concentrations produced a marked inhibition.

$MgCl_2$ increased the activity of the EDTA-treated preparations by several fold. The optimum concentrations were found to be 80 mM, 2.5 mM and 20 mM for the bilirubin, *p*-nitrophenol and phenolphthalein reactions, respectively. The increase in activity by these concentrations was 5, 2.8 and 2.2 times for each of the three substrates. The assaying mixtures were designed according to these requirements. Other compounds were tested for their effect on the transfer reaction and the results are shown in Table VI.

The conjugation of bilirubin with glucuronic acid was not affected by diphenylacetic acid at concentrations ranging from 0.08 to 0.4 mM or by *p*-nitrophenol at concentrations between 0.2 and 0.8 mM. Conversely, bilirubin did not affect *p*-nitrophenol conjugation at concentrations between 0.04 and 0.4 mM. Phenolphthalein inhibited bilirubin and *p*-nitrophenol conjugation and in both instances the inhibition was of the non-competitive type. Fig. 5 shows the inhibition of bilirubin conjugation by 0.064 mM phenolphthalein. The data were plotted according to LINEWEAVER AND

BURK[13]. On the other hand, phenolphthalein conjugation was not affected by *p*-nitrophenol or by bilirubin. The conjugation of phenolphthalein with glucuronic acid was inhibited by phenolphthalein itself at relatively low concentrations. When rat microsomes were used, the rate of the reaction increased up to a concentration of 0.5

TABLE VI

EFFECT OF DIFFERENT COMPOUNDS ON UDP-GLUCURONYLTRANSFERASE ACTIVITY

Additions	*Acceptor substrate*	
	p-Nitrophenol (units/ml)	*Bilirubin (units/ml)*
None	1000	159
10^{-3} M UMP	914 (91%)	111 (70%)
10^{-3} M UDP	771 (77%)	102 (64%)
10^{-3} M iodoacetate	1028 (103%)	149 (94%)
10^{-3} M hydroxymercuribenzoate	400 (40%)	41 (26%)
10^{-3} M glucuronic acid	822 (82%)	170 (107%)
$1.2 \cdot 10^{-3}$ M deoxycholate	600 (60%)	13 (8%)

mM and then decreased sharply to be undetectable at a concentration of 3 mM. Microsomal preparations from rabbits showed a similar behavior. Maximum activity was found at phenolphthalein concentrations of 0.3 mM and complete inhibition at 0.75 mM. The conjugation of *p*-nitrophenol and bilirubin did not show this behavior and saturating concentrations for rat microsomes were 2.2 mM and 0.32 mM, respectively.

β-Glucuronidase assays

Rat-liver homogenates liberated phenolphthalein from phenolphthalein glucuronide at a rate of 0.5 μmole per g of liver tissue in 10 min under the conditions

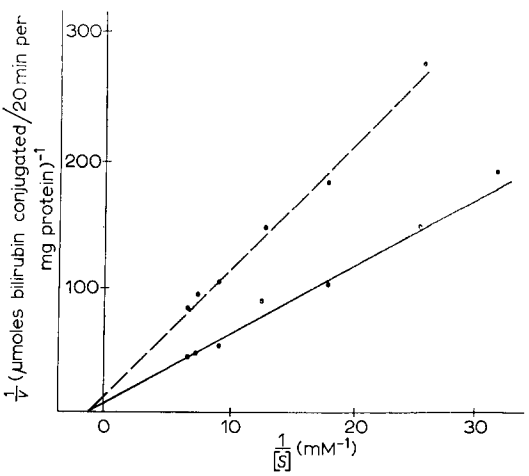


Fig. 5. Lineweaver–Burk plot showing bilirubin conjugation in the presence of phenolphthalein (— — —) and in the absence of it (—).

described under METHODS. After dialysis against EDTA for 18 h the activity remained unchanged or increased slightly.

Chromatography of bilirubin conjugation products

Two spots were visible that had R_F values of 0.51 and 0.33.

Purification

The UDP-glucuronyltransferase activity towards bilirubin was purified by about 6 fold. Solubilization by deoxycholate resulted in the loss of about 50% of the activity. A flow sheet representing our results is shown in Table VII.

Stability

A microsomal preparation lost 20% of its activity towards *p*-nitrophenol and bilirubin in 6 days and 70% in 15 days. A deoxycholate-solubilized enzyme was found to have 50% of the original activity after 5 days. Storage was at 3°.

TABLE VII

SOLUBILIZATION OF UDP-GLUCURONYLTRANSFERASE FROM RAT LIVER

Enzyme fraction	*Total protein (mg)*	*Total bilirubin units*	*Specific activity (units/mg protein)*	*Total p-nitrophenol units*	*Specific activity (units/mg protein)*
Liver homogenate	2636	18 036	6.8	78 170	29.6
2000 × *g* for 15 min, supernatant	2180	15 336	7.0	74 230	34.1
Ammonium sulfate, 0–42% fraction	766	15 660	20.4	56 243	73.4
Washed microsomes	269	12 744	47.4	51 390	191.0
Deoxycholate solubilized enzyme	260	6 696	25.8	21 698	83.4

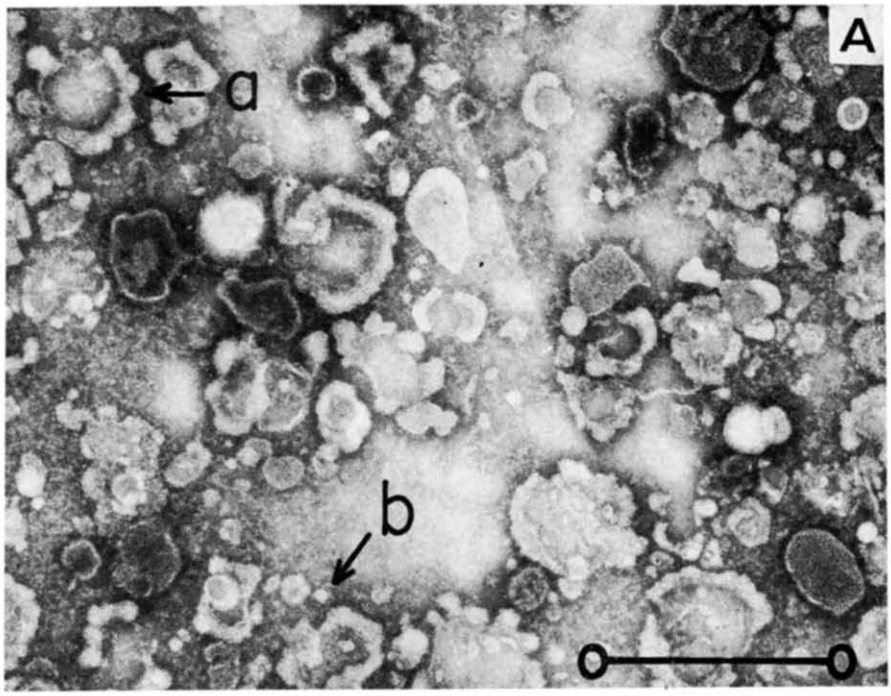
The optimum pH for the reaction towards *p*-nitrophenol and bilirubin was found to be between 7.8 and 8.0.

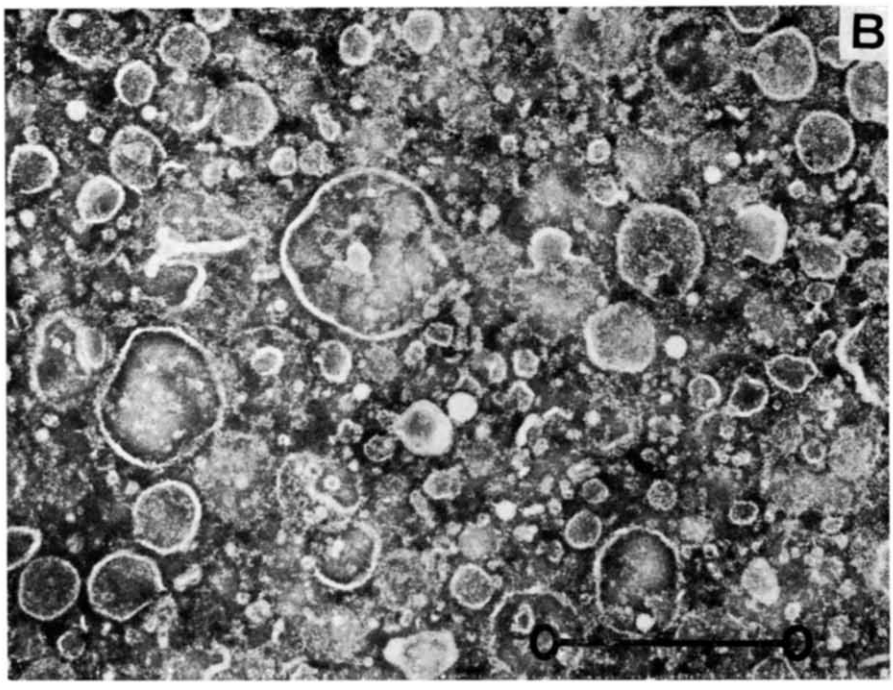
Electron microscopy

The microsomal pellets which had not been treated with EDTA or deoxycholate showed membranous structures with the shape of vesicles (Fig. 6A). The external aspects of these vesicles were irregular and showed protuberances that measured about 500 Å (arrow a), giving to the vesicles the appearance of rosettes. There were round particles scattered throughout the preparations with a size of approx. 300 Å (arrow b).

In the microsomal pellets treated with EDTA the vesicular structures were still present but the membranes had lost their rough appearance and the inner and outer aspects were equally smooth. The round particles measuring 300 Å were present although examination of many fields seemed to indicate a decrease in their number. There was also the appearance of a background of amorphous material (Fig. 6B).

The pellets treated with EDTA and then with deoxycholate revealed the fragmentation of some of the membranous structures described above and a marked decrease in the number of the round, ribosome-like structures (Fig. 6C).


A
a
b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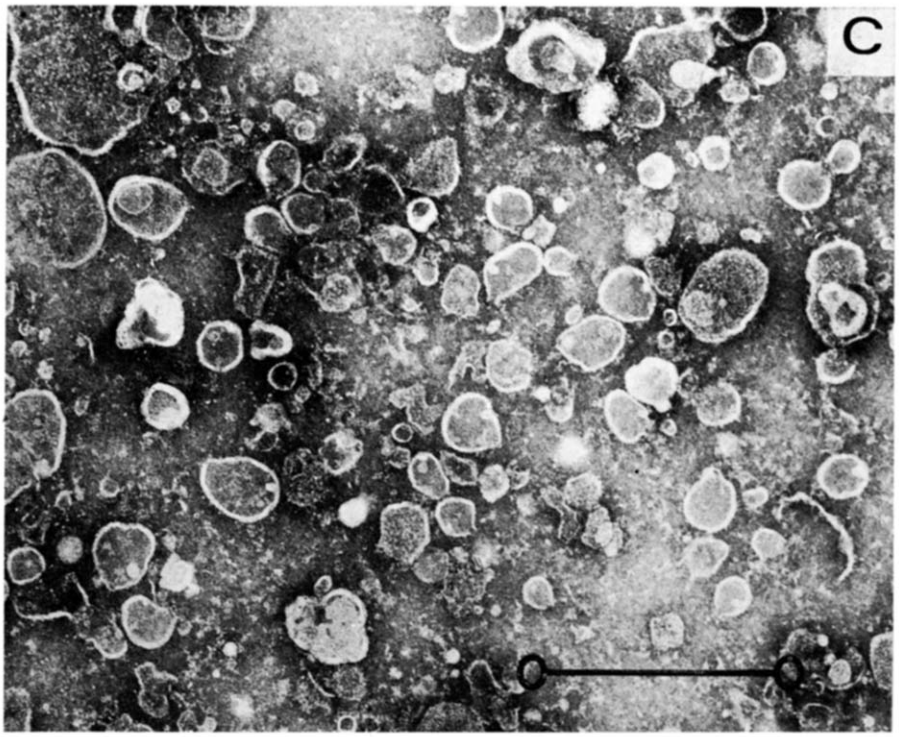

Fig. 6. Electron microscope pictures of microsomal pellets: A, before EDTA; B, after EDTA; C, after EDTA and deoxycholate. Final magnification is 60 000 (0.5 μ = 3.0 cm, ○—○, as drawn in the photographs).

DISCUSSION

The first description of the conjugation of bilirubin with glucuronic acid *in vitro* is almost 10 years old[3]. Many other substrates are conjugated under similar conditions, but these diverse substrates may share no biological property other than that of being excreted after glucuronidation. The need for elimination of some of these compounds may be critical at some stage of development (*e.g.* the elimination of bilirubin during the neonatal period), so the need for establishing the simplicity or complexity of this system is important not merely from the standpoint of biochemical characterization. But the last must come first if a meaningful evaluation of the UDP-glucuronyltransferase system is attempted.

Different investigators have examined the GlcUA transfer reaction in detail. POGELL AND LELOIR[14] and ISSELBACHER, CHRABAS AND QUINN[15] have contributed important data on the conjugation of *p*-nitrophenol with GlcUA and one of us has presented data on the formation of phenolphthalein glucuronide[7]. TOMLINSON AND YAFFE have recently published their results on the developmental and kinetic aspects of UDP-glucuronyltransferase[16] using bilirubin and *p*-nitrophenol as substrates and concluded that their data was compatible with the existence of more than one enzyme. The conjugation of bilirubin *in vitro* has been described by a number of other workers who have used liver homogenates or liver slices[1,17–19]. The rates of conjuga-

tion of bilirubin observed by these investigators fall short of the known capacity of the liver to eliminate bilirubin *in vivo*. This capacity has been measured by BILLING, MAGGIORE AND CARTER[4] in rats. The maximum hepatic clearance value found was 54 ± (S.E.) 1.81 μg per 100 g body weight per min. If we assume that the liver of these animals represents 3.0% of their body weight[20] bilirubin is being eliminated at a rate of 540 μg per g liver per 30 min (0.93 μmole per g liver per 30 min). Since bilirubin excretion is dependent on its previous conjugation with GlcUA and this conjugation, at least in the adult animal, seems to take place in the liver[21], we can assume that the value measured by BILLING, MAGGIORE AND CARTER[4] represents conjugation of bilirubin with glucuronic acid by the liver. Similar calculations based on the data obtained by SERENI *et al.* from liverperfusion experiments[22] give the rat liver a capacity to form bilirubin glucuronide in the range of 200 μg per g liver per 30 min, and the highest figure reported *in vitro* (except with perfusion) is 115 μg per g per 30 min (ref. 3). From a comparison of these results one can conclude that the *in vitro* systems are somewhat vitiated by the manipulations to which they were subjected, or that the conditions used for the measurement of bilirubin glucuronide synthesis are not optimal, or both together.

We have now found that dialysis of liver fractions against alkaline EDTA produces a marked activation of the rate at which these fractions conjugate bilirubin with glucuronic acid. The rate measured under the conditions described under METHODS is similar to the maximum hepatic clearance found in experiments *in vivo*.

The measurement of the conjugation of bilirubin with GlcUA as described here has been shown to be a very reliable and reproducible procedure, more so than the determination of *p*-nitrophenol and phenolphthalein glucuronide synthesis, where the determination is based in the disappearance of color. When bilirubin is the substrate, what is measured is the appearance of color, which makes this method more sensitive and reliable than the other two.

The mechanism by which EDTA at alkaline pH activates UDP-glucuronyltransferase remains to be elucidated. As has already been remarked, the activation is not immediate. Under our conditions for the assay of *p*-nitrophenol (5–10 minutes incubation) we could find no activation when EDTA was added to an assay system containing undialyzed liver microsomes. There was, instead, a 16% inhibition. This result is in disagreement with those reported by POGELL AND LELOIR[14] and by HUTTUNEN AND MIETTINEN[23]. But the discrepancy is only apparent, since we have found a 49% stimulation of this reaction when the incubation time is prolonged to 30 min as in the experiments of these investigators. With the activated samples this prolonged incubation time is neither necessary nor desirable.

The activation by EDTA may be due to a number of factors, and HUTTUNEN AND MIETTINEN have made an analysis of some of them[23]. One such mechanism, as described by POGELL AND LELOIR[14], is the inhibition of pyrophosphatase activity that breaks down UDP-GlcUA. We have not been able, however, to increase the rate of conjugation by increasing the concentration of UDP-GlcUA in the assaying mixture. We do not believe that the activation found by dialysis against EDTA is due to solubilization, since the fraction solubilized by the first EDTA dialysis is about 20% in the rabbit and only 10% in the rat, while the activation observed by us is of the order of 5 fold. Although subsequent washes with EDTA result in further solubilization, this does not result in increased activity. Part of the activation is dependent on

the pH at which the dialysis is performed, since dialysis against a glycine solution at pH 9.0 produces a significant activation[7]. Dialysis against EDTA produced no change in the activity of β-glucuronidase nor any increase in its activity. This precludes any implication of this enzyme in the activation of UDP-glucuronyltransferase.

The activation of UDP-glucuronyltransferase is concomitant with the fragmentation of some of the structures that constitute the microsomal fraction. This is clearly visible by the presence of a fatty layer in the upper part of the centrifuge tube after the EDTA-treated microsomes are centrifuged. The examination of the pellet material by electron microscopy showed that the most apparent change induced by EDTA was the transition from rough to smooth membranes. Since a significant number of ribosome-like structures still remained after the dialysis against EDTA it was not possible to decide to which structure, membranes or ribosomes, UDP-glucuronyltransferase(s) is bound, or if there is such a partition. The structures that remained after EDTA–deoxycholate treatment were mostly membranes and this pellet was particularly rich in activity with respect to *p*-nitrophenol. It should be pointed out that by raising the deoxycholate concentration in the solution which is used to homogenize the EDTA-treated microsomes, the sedimentable material disappears. It is conceivable that there are different types of membrane structures in the microsomal fraction with different sensitivities to the disruptive effect of deoxycholate.

The activation of UDP-glucuronyltransferase here described is similar to the activation of "latent" ribonuclease and deoxyribonuclease described by ELSON[24] when he disrupted *E. coli* ribonucleoprotein particles with different procedures. NEU AND HEPPEL[25] found later that "latent" ribonuclease in *E. coli* is found in the space between wall and cell membrane in an active form. To demonstrate the presence of active ribonuclease in this localization the bacterial cell wall had to be disrupted without altering the integrity of the protoplast. The implication of this finding is that latent ribonuclease is a free and not a particulate enzyme and that it becomes inactive by its attachment to the ribosomal particles during extraction procedures that do not maintain the integrity of the protoplast. A similar mechanism could conceivably occur in systems like liver extracts, and a soluble or particulate enzyme could become inactive or latent by a change or disruption of the normal environmental conditions.

Deoxycholate was found to solubilize UDP-glucuronyltransferase from microsomal pellets that had been treated with EDTA. The combination of these two agents afforded soluble preparations that contributed data in favor of the existence of an UDP-glucuronyltransferase for bilirubin different from the enzyme that catalyzes the formation of *p*-nitrophenol glucuronide.

Centrifugation of the activated microsomes after they were homogenized in the deoxycholate solution showed that the activity responsible for the formation of *p*-nitrophenol glucuronide was concentrated in the pellet while the bilirubin conjugase activity was uniformly distributed through the length of the tube. This indicates that the UDP-glucuronyltransferase that conjugates bilirubin with GlcUA is bound to structures that are completely disrupted at the deoxycholate concentration used in our experiments, while the activity towards *p*-nitrophenol seems to be mostly attached to structures less sensitive to the action of deoxycholate.

The heat-inactivation studies showed that the activity towards bilirubin was more stable under the conditions we used and that the optimum pH for stability of the two activities was different. Unless one postulates that the active site of a single

enzyme is changed in such a way that affinity for different substrates are affected differently, the conclusion seems inescapable that there should be two active sites. The binding of two unrelated chemical compounds by an enzyme has been described for those enzymes susceptible to allosteric repression[26]. The binding site for the repressor molecule has been shown to be more sensitive than the binding site for the substrate molecule and can be selectively inactivated. However, this model can hardly be compared with UDP-glucuronyltransferase since an allosteric repressor is not a substrate for the enzyme that it represses.

The elution pattern from a Sephadex G-200 column showed the elution of UDP-glucuronyltransferase activity towards *p*-nitrophenol and bilirubin in the same fractions. Although no separation of the two activities was found, the pattern of elution was somewhat different, and the difference was reproducible.

Kinetic studies showed no inhibition between *p*-nitrophenol and bilirubin. These results are in partial disagreement with those reported by TOMLINSON AND YAFFE[16]. Phenolphthalein was, however, a non-competitive inhibitor of bilirubin conjugation. Phenolphthalein is conjugated with GlcUA through the phenolic groups, but the molecule has a carboxyl group that could conceivably interfere with the activity of UDP-glucuronyltransferase towards bilirubin.

During the last few years evidence has been accumulating in favor of the existence of more than one UDP-glucuronyltransferase. The Gunn rat, which is unable to synthesize bilirubin glucuronide, eliminates aniline glucuronide in a normal fashion[27], and is capable of conjugating *p*-nitrophenol with GlcUA (ref. 28). JAVITT has recently reported that the Gunn rat is also capable of eliminating diphenylacetic acid–GlcUA as efficiently as control rats[29]. This observation is particularly interesting because the conjugation of GlcUA to diphenylacetic acid is effected through an acyl bond, as is the case with bilirubin. The findings reported by JAVITT are in agreement with the lack of inhibition that we found between bilirubin and diphenylacetic acid.

LEVITZ* has recently studied a patient with the Najjar–Criggler syndrome, the human equivalent of the Gunn rat, for his capacity to eliminate estriol. Although this patient was deeply icteric and his serum bilirubin was of the indirect type, he was found to be capable of promptly eliminating [^{3}H]estriol; 85% of this was as estriol glucuronide and the 16-glucuronide predominated. Finally, Dutton has reported that newborn rats, which are unable to conjugate bilirubin, were capable of forming *p*-nitrophenol glucuronide. YAFFE has obtained similar results[16].

We believe our data contribute further evidence in favor of the existence of different UDP-glucuronyltransferases and provide methods of evaluating the conjugation of bilirubin under conditions that yield results compatible with the information we have about bilirubin excretion from experiments *in vivo*.

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* M. LEVITZ, personal communication.

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