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PARTIAL PURIFICATION OF HEPATIC UDP-GLUCURONYLTRANSFERASE

STUDIES OF SOME OF ITS PROPERTIES

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SUMMARY

1. Purification of UDP-glucuronyltransferase (UDP-glucuronate glucuronyltransferase, EC 2.4.1.17) from guinea pig liver, achieved by ultrasonication, sucrose gradient fractionation and Sephadex G-200 elution, increased the capacity to conjugate bilirubin *in vitro* 20-fold and *o*-aminophenol 50-fold. The stability of enzymatic activity in this preparation was greater than previously described for this enzyme in preparations with a comparable increase in initial activity and was strikingly similar with both substrates. The purification procedure did not separate conjugating ability for any of the substrates tested.

2. The inadequacy of gravitational criteria alone in indicating solubilization of a membrane-bound enzyme was demonstrated by electron microscopy. Methods used unsuccessfully in attempting true solubilization are listed.

3. The purified enzyme preparation was shown to contain a number of proteins including microsomal enzymes and lipids. The possible effect of these on kinetic studies is critically discussed.

4. Application of the purification procedure to livers of cat, Gunn rat and hypophysectomized or thyroidectomized rats also yielded a stable purified enzyme preparation, without separating conjugating ability for the substrates tested.

5. The results of these investigations are discussed and their relationship to possible multiplicity of UDP-glucuronyltransferase considered.

INTRODUCTION

UDP-glucuronyltransferase (UDP-glucuronate glucuronyltransferase, EC 2.4.1.17) is primarily a hepatic microsomal enzyme which catalyzes the transfer of glucuronic acid from UDP-glucuronic acid to a variety of hydroxyl, carboxyl, amino[1–3] and probably sulfhydryl acceptors[4]. Various drugs[5] as well as bilirubin[6], thyroxine[7] and some steroid hormones[7] are conjugated by this enzyme system.

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The broad substrate spectrum, varied rates of development of UDP-glucuronyltransferase activity with different substrates in different species[8], and the ability of the cat and Gunn rat[9] to form glucuronides with some substrates but not others[10,11], have caused some investigators[10,12] to suggest that there may be multiple forms of the enzyme which vary from species to species. The relative ease of solubilization of enzymatic activity for some substrates[13–15], the distribution of conjugating capacity with different substrates in rough and smooth microsomal preparations[16], as well as evidence from kinetic studies[13,14,17] have been interpreted also as indicating that the enzyme has multiple forms. But each of these investigations was performed using homogenate, microsomal or unstable partially purified solubilized enzyme preparations.

The question of multiplicity is an important one. While it remains unanswered, doubts must persist about the relevance of studies done with laboratory animals and easily measured substrates to such practical problems as the role of the apparent low activity of this enzyme in the etiology of "physiological" jaundice in the human newborn[18].

The present studies were undertaken to obtain a soluble stable purified preparation which might resolve the problem of multiplicity. UDP-glucuronyltransferase was partially purified in a more stable form than has hitherto been reported. Purification did not separate the ability to form glucuronides with the different acceptors examined. In addition, the study illustrates that gravitational methods alone do not permit the conclusion that a membrane-bound enzyme has been made soluble.

Part of this study has been briefly reported[19].

MATERIALS AND METHODS

Materials

UDP-glucuronate as ammonium salt, crystalline bilirubin and human serum albumin, all stated to be 98–100% pure, were obtained from Sigma Chemical Co. Ltd., St. Louis, Mo., U.S.A., as were *Trimeresurus flavoviridis* and *Naja naja* snake venoms. *o*-Aminophenol was resublimed from commercially available *o*-aminophenol. *o*-Aminophenol glucuronide was a gift from Professor R. T. Williams, St. Mary's Hospital, London. "Bilirubin glucuronide" purified from bile was obtained from Professor C. J. Watson, University of Minnesota, Minneapolis, Minn.

$(NH_4)_2SO_4$ (Merck) was recrystallized twice from water. All other chemicals were obtained from commercial sources in the highest purity available and were not further purified.

Animals

The following male animals were maintained on lab chow and water *ad libitum* without starvation prior to their use in experiments:

Hartley guinea pigs (Hazleton Research Animals, Md.), Wistar rats (Maryland Lab., N.J.), Gunn rats (own colony) and normal, thyroidectomized and hypophysectomized Sprague–Dawley rats (Charles River Laboratories, Mass.). Hypophysectomy and thyroidectomy were performed by Charles River Laboratories in immature male rats at least 2 months before these animals were sacrificed. Both operations were judged effective if the animals failed to maintain a normal rate of growth. Thyr-

oidectomized animals required 1% calcium lactate in the drinking water to prevent hypocalcaemia.

UDP-glucuronyltransferase activity

Assays were performed at 37°, using previously described techniques with *o*-aminophenol[20], anthranilic acid[21], *p*-nitrophenol[22] and methylumbelliferone[23] as glucuronide acceptors, incubations lasting 20, 20, 10 and 30 min, respectively. Bilirubin conjugation was measured using the technique of VAN ROY AND HEIRWEGH[24] in the presence of triethanolamine–HCl (pH 7.4) with 340 μM bilirubin and 130 μM human serum albumin which was dialyzed against 0.001 M EDTA (pH 7.4). Incubation time was 40 min. Anthranilate and *p*-nitrophenol glucuronide concentration was standardized using the respective substrate. *o*-Aminophenol glucuronide and bilirubin glucuronide were standardized against the appropriate glucuronide, which in the case of bilirubin glucuronide had been quantitated by the MALLOY AND EVELYN[25] technique. In all assays optimum amounts of UDP-glucuronate were present. With each tissue examined, the amount of enzymatically active protein added was adjusted to give adequate absorbances while maintaining initial velocities. *o*-Aminophenol and bilirubin were used as substrates in most studies, since with these acceptors glucuronide formation is measured directly in the presence of excess substrate. Furthermore they represent a hydroxyl and carboxyl acceptor, respectively. In kinetic studies for K_m determination, incubation was for 10 min with *o*-aminophenol and 45 min with bilirubin, as these times gave absorbances in the assay tubes significantly above the blank values while maintaining initial velocity conditions.

Nucleoside phosphatase activity was determined by the method of NOVIKOFF AND HEUS[26] and glucose-6-phosphatase activity as described by DE DUVE *et al.*[27].

Protein was determined by the method of LOWRY *et al.*[28] except in the eluate from Sephadex columns where absorption at 280 mμ was determined using a DU Beckman spectrophotometer and a 1-cm light path.

Electron microscopy: 1 drop of the Sephadex G-200 eluate was added to 1 drop of phosphotungstic acid (1%) buffered to pH 7.0 with KOH, and transferred to a Formvar (3%) carbon-coated grid, on which it was visualized using a Siemen's Elmiscope 1A electron microscope at 80 kV.

Specific density of sucrose-containing solutions was determined by measuring the refractive index (Abbe 3L Refractometer, Bausch and Lomb, Rochester, N.Y. 14602) from which the specific density was derived from published data[29].

Preparation of enzyme

Animals were killed by cervical dislocation. Guinea pigs weighing 200–400 g were used for all studies, except where noted. The liver was removed rapidly and all subsequent procedures performed at 0–4°. A 25% (w/v) homogenate in 0.14 M KCl, 0.01 M Tris–HCl (pH 8.0) was prepared using a mechanical glass–Teflon homogenizer (A. H. Thomas and Co., Philadelphia, Pa.) centrifuged at 2000 $\times g$ for 15 min, and the total particulate fraction was sedimented from the supernatant by centrifugation at 100 000 $\times g$ for 45 min. The particulate fraction was resuspended in the homogenizing buffer to a volume equal to the volume of the initial supernatant and brought to 42% saturation with saturated $(NH_4)_2SO_4$ (pH 8.0) containing 1.0 ml conc. NH_4OH and 1 mM EDTA per l. After 1 h, the precipitate was sedimented by centrifugation

and resuspended in the homogenizing solution to the same volume as the particulate suspension. This suspension was dialyzed against three changes of 50 vol. of 0.01 M EDTA, 0.2 mM mercaptoethanol (pH 9.0) for 18 h. The dialyzed particulate fraction, prepared as described for a homogenate suspension by HALAC AND REFF[14], lost 30–40% of enzymatic activity in the first 7 days and was used as the starting material for attempts to solubilize enzymatic activity.

Enzyme solubilization

In the preparation of a purified enzyme, solubilization was considered a necessary first step. Published studies with UDP-glucuronyltransferase have accepted as evidence of solubilization the fact that enzymatic activity is found in the supernatant after centrifugation at greater than 80 000 × *g* for 30 min or more[10]. In this study, solubilization was assumed when enzymatically active material was found in the supernatant after 45 min centrifugation at 100 000 × *g* (av.).

By ultrasonic oscillation, LEVENTER *et al.*[30] solubilized UDP-glucuronyltransferase from guinea pig liver microsomes. Using a Branson Sonic Power Sonifer (Branson Sonication, Danbury., Conn.) ultrasonic oscillation at 7 A for 20 periods of 10 sec each at 40-sec intervals while maintaining the temperature at less than 3° caused no loss of enzymatic activity. Up to 40% of enzymatic activity in the dialyzed particulate fraction was recovered in the translucent pale yellow supernatant obtained after centrifugation at 100 000 × *g* for 45 min. This fluid was withdrawn using a bent-tipped pasteur pipette, leaving a small decantable deposit, Sediment A, and a white pellet, Sediment B, which was resuspended in 0.14 M KCl, 0.01 M Tris (pH 8.0) for use in enzyme assays. The results of a typical experiment are shown in Table I. At 5°, more than 60% of the original enzymatic activity in the supernatant remained after 7 days.

With anthranilate as glucuronide acceptor and using 0.3% deoxycholate as a solubilizing agent, we corroborated the findings of HALAC AND REFF[14] but did not consider that this method of solubilization could be exploited further as the inhibitory effect of deoxycholate on UDP-glucuronyltransferase activity required that it be removed after any protein fractionation prior to determination of enzymatic activity. The observation that deoxycholate at a concentration which stimulates enzymatic

TABLE I

EFFECT OF ULTRASONIC OSCILLATION ON UDP-GLUCURONYLTRANSFERASE ACTIVITY AND DISTRIBUTION ON CENTRIFUGATION

Dialyzed particulate fraction was sonicated, centrifuged at 100 000 × *g* for 45 min and separated into supernatant, Sediment A and Sediment B, as described in the text. Enzymatic activity, determined using *o*-aminophenol as glucuronide acceptor, is expressed as nmoles of *o*-aminophenol glucuronide formed per mg protein in 20 min.

Enzyme preparation	*Total enzymatic activity*	*Percentage activity*	*Activity per mg protein*
Dialyzed particulate fraction	3140	100	53
Uncentrifugated sonicated material	2970	95	53
Supernatant	1046	33	47
Sediment A	1620	52	92
Sediment B	495	15	92

TABLE II

METHODS USED IN ATTEMPTING SOLUBILIZATION OF UDP-GLUCURONYLTRANSFERASE FROM DIALYZED PARTICULATE FRACTION

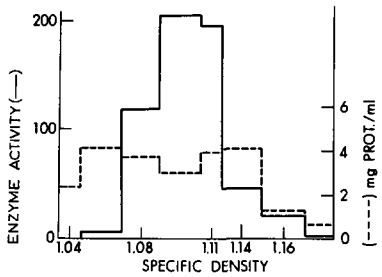
Methods resulting in total loss of enzymatic activity	*Methods resulting in partial loss of activity and no solubilization*	*Methods resulting in partial loss of activity and slight solubilization*	*Methods resulting in neither loss of activity nor solubilization*
33% pyridine[32]	6 M urea followed by dialysis against 0.01 M EDTA and 1 mM mercaptoethanol	1.5% acetic acid for 1 min[39]	3 M NaCl
Sulfonation in the presence of 8 M urea and L-cysteine, with subsequent excess mercaptoethanol[33]	Incubation with heat-treated venom from *T. flavoviridis*[35] and *Naja naja*[36]	0.1% Triton[40]	
0.1 M sodium acetate (pH 4–5.5)	Incubation with trypsin and chymotrypsin[37]	0.1% Nonidet P40	
50% dimethyl formamide	0.05% lysolecithin[38]		
1.5% Triton and 2.6 M urea[34]	Freezing and thawing		
1 M hydroxylamine sulfate (pH 9.0)	20% dimethyl sulfoxide		
0.1% octyl sodium sulfate			
0.1% decyl sodium sulfate			
0.1% sodium desoxytaurocholate			

activity in freshly prepared tissue preparations is inhibitory at the same concentration after the tissue had been stored at 0° (ref. 31) also suggested its unsuitability for use in a prolonged purification procedure. Solubilization was attempted using a number of methods under a variety of circumstances with little success. These studies are summarized in Table II.

In addition we attempted to increase the efficiency of solubilization produced by ultrasonic oscillation by using it in combination with other procedures. Sonication in the presence of 3 M NaCl or 4 M urea or the addition of these agents to sonicated dialyzed microsomal fraction did not increase the release of enzymatic activity into the supernatant. Freezing and thawing of the dialyzed microsomal fraction before or after ultrasonic oscillation did not enhance solubilization. When sonicated dialyzed microsomal fraction was incubated with heat-treated, *T. flavoviridis* venom[35] or made 0.05% with lysolecithin, no increase in solubilization occurred.

Enzyme purification

Following ultrasonic oscillation and centrifugation at 100 000 $\times g$, the supernatant from 30 ml of dialyzed microsomal fraction was concentrated to 2.5 ml by ultrafiltration (using Diaflo, UM 10 filter, Amicon Corp., Lexington, Mass.) and placed on a 25-ml continuous sucrose gradient (13–46%) in 0.05 M Tris and 1.0 mM EDTA (pH 8.0). The gradient was centrifuged in a Spinco SW-25 swinging-bucket rotor at 25 000 rev./min (53 500 $\times g$ (av.)) for 19 h and subsequently fractionated. As illustrated in Fig. 1, protein concentration was uniform below specific density 1.14, while enzymatic activity was concentrated in the fraction with specific density greater than 1.08 and less than 1.11. This fraction was concentrated to 2.0 ml and eluted from a Sephadex G-200 column (2.5 cm in diameter and 35 cm in length) with 0.01 M Tris and 1 mM



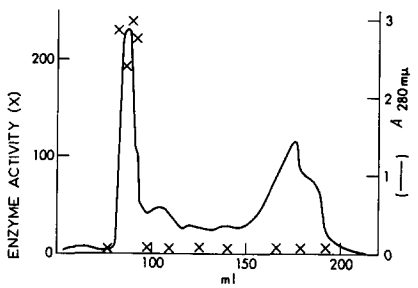


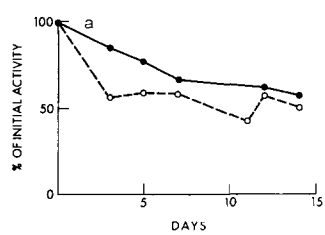
Fig. 1. Distribution of UDP-glucuronyltransferase on a sucrose gradient. Concentrated supernatant fluid, obtained following ultrasonic oscillation of dialyzed particulate fraction, was added to a continuous sucrose gradient which was centrifuged at 53 000 × *g* (av.) for 19 h and subsequently fractionated. UDP-glucuronyltransferase activity is expressed as nmoles *o*-aminophenol glucuronide formed per mg protein.

Fig. 2. Elution of UDP-glucuronyltransferase from Sephadex G-200. Enzymatic activity is expressed as nmoles *o*-aminophenol glucuronide formed per mg protein per assay. Protein concentration was estimated by determination of absorbance at 280 mμ in each sample as well as by the method of LOWRY *et al.*[28]. The pattern of protein elution as determined by the two methods was similar.

EDTA (pH 8.0) at 16 ml/h. As shown in Fig. 2, enzymatic activity was limited to the large protein peak eluted with the void volume. Recovery of enzymatic activity was 100%.

Table III is a flow sheet for the complete purification procedure in an experiment in which UDP-glucuronyltransferase activity was assayed using *o*-aminophenol and bilirubin as substrates. In this experiment, *o*-aminophenol glucuronide formation increased 32-fold, while bilirubin glucuronide formation increased 15-fold. In the best preparations, the increase in activity amounted to 50- and 20-fold, respectively. At no point in purification was the capacity to form *o*-aminophenol glucuronide separated from the capacity to form bilirubin glucuronide. Conjugation of anthranilic acid and *p*-nitrophenol also increased by a factor of 20–30 during purification. Methylumbelliferone was conjugated by the purified enzyme preparation, but this substrate was not studied further.

Additional efforts were made to obtain a more purified enzyme preparation from the supernatant from sonicated dialyzed particulate fraction. Triton, dimethylsulfoxide or urea was added to this fluid, after it had been concentrated as before, to give final concentrations of 0.1%, 20% and 4 M, respectively, before elution from



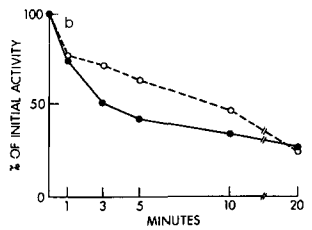


Fig. 3. Stability of UDP-glucuronyltransferase in Sephadex G-200 eluate. Using *o*-aminophenol (●) and bilirubin (○) as substrates, enzymatic activity expressed as percentage of initial activity was determined after storage (a) at 4° and (b) at 45°.

TABLE III

PURIFICATION OF UDP-GLUCURONYLTRANSFERASE FROM GUINEA PIG LIVER

Substrate		*Enzyme preparation*			
		25% homogenate	*Total particulate suspension*	*Dialyzed particulate fraction*	*Sephadex G-200 eluate*
	Volume (ml)	188	90	60	28
	Total protein (mg)	9 400	1 620	660	88
o-Aminophenol	Total conjugated (nmoles)	75 000	46 000	56 000	23 000
	% Initial activity	100	61	75	30
	nmoles conjugated per mg protein	8	28	85	259
	Increase in initial activity		3.5	11	32
Bilirubin	Total conjugated (nmoles)	7 500	4 050	4 760	1 140
	% Initial activity	100	54	63	15
	nmoles conjugated per mg protein	0.8	2.5	7.2	13
	Increase in initial activity		3	9	14

Sephadex G-200, which had been equilibrated with these agents in 0.01 M Tris and 1 mM EDTA (pH 8.0). The protein elution pattern and localization of enzymatic activity is as illustrated in Fig. 2. These parameters were also unchanged when the fraction from the sucrose gradient was sonicated again before elution from Sephadex G-200 equilibrated with 0.01 M Tris and 1 mM EDTA (pH 8.0). These procedures did not increase enzyme purification. Attempts to further purify the enzyme preparation obtained by sucrose gradient fractionation and Sephadex G-200 elution by $(NH_4)_2SO_4$ fractionation or by elution from either DEAE-cellulose or CM-cellulose under a variety of conditions were unsuccessful.

Stability of enzymatic activity

Stability of enzymatic activity in the Sephadex G-200 eluate was investigated at 4 and 45° using *o*-aminophenol and bilirubin as substrate. With either substrate, more than 50% of the original activity remained after storage at 4° for 7–14 days, while 30% of activity was retained after heating at 45° for 20 min (Figs. 3a and 3b). With *p*-nitrophenol as substrate, 40% of the original activity was retained after heating for 20 min at 45°.

Physicochemical characteristics of the enzymatically active Sephadex G-200 eluate

A number of investigations were undertaken to determine the physicochemical nature of the enzymatically active material. Electron microscopy revealed small membranous structures, often in the form of vesicles, of 800–2000 Å in diameter, some of which were collapsed giving an apparent double membrane (Fig. 4). These structures were not seen in aliquots of the Sephadex G-200 eluate which had similar protein concentration but no enzymatic activity.

The lipid content was examined after extraction by the technique of FOLCH

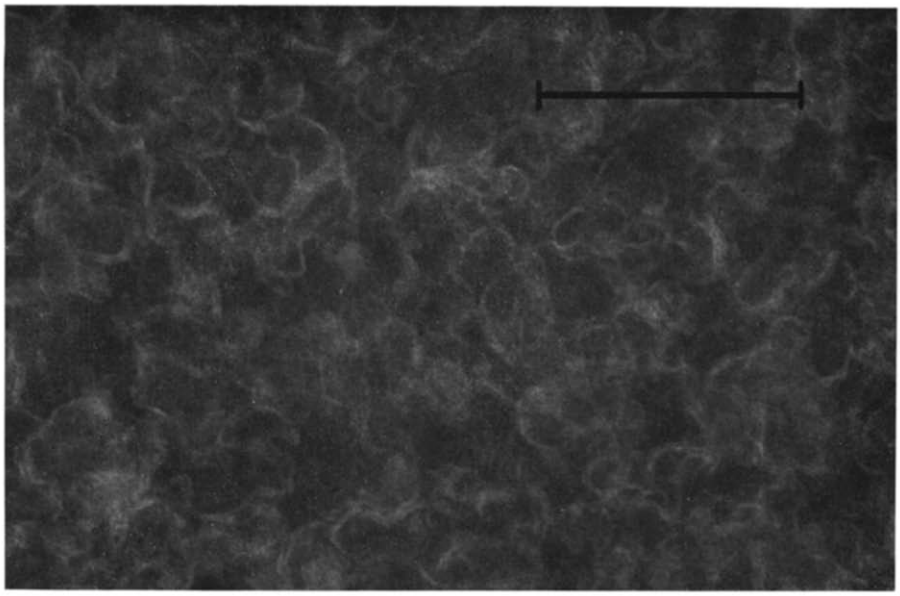


Fig. 4. Electron microscopic appearance of enzymatically active Sephadex G-200 eluate. All fields examined contained such structures which were not present in samples of eluate with similar protein content but no enzymatic activity. Final magnification is 70 000 ×. Bar represents 0.5 μ = 3.5 cm.

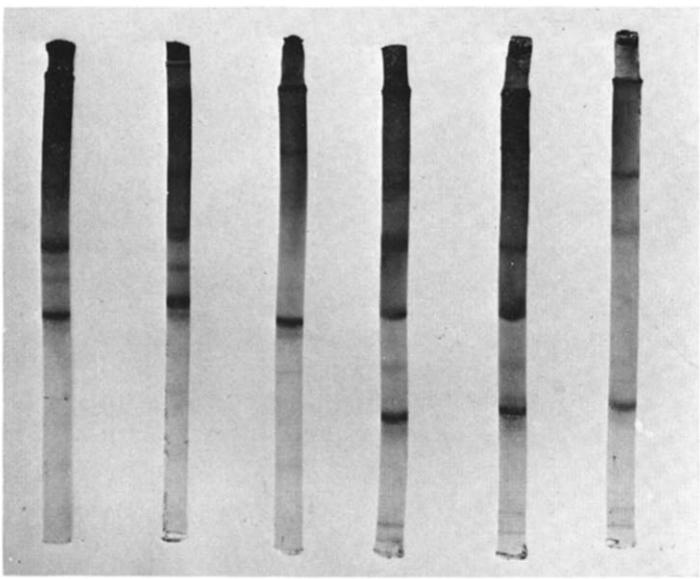


Fig. 5. Electrophoresis of particulate suspension, dialyzed particulate fraction and Sephadex G-200 eluate. Polyacrylamide gel electrophoresis of particulate suspension, dialyzed particulate fraction and Sephadex G-200 eluate. From left to right: Gel 1, particulate suspension; Gel 2, dialyzed particulate fraction; Gel 3, Sephadex G-200 eluate all made 8 M urea in 0.05 M K_2CO_3; Gels 4, 5 and 6 were similar to Gels 1–3 but 0.1 vol. mercaptoethanol was also added to the protein. Gels were 8 M urea prepared in Tris–HCl (pH 8.4). Electrophoresis ran from cathode to anode in Tris–glycine buffer (pH 8.7) for 4 h at 5 mA per gel. Gels were fixed with 20% sulfasalicylic acid and stained with 7% Coomassie Blue and destained with 7% acetic acid.

TABLE IV

LIPID CONTENT OF PURIFIED ENZYME PREPARATION

After extraction, the lipid content was determined by thin-layer chromatography and chemical analysis[43]. Reported values for hepatic microsomes are given for comparison[42].

	Enzyme preparation	*Hepatic microsomes*
Phospholipid/protein	0.75	0.4
Cholesterol/phospholipid	0.03	0.12
Phospholipid distribution (%)		
Phosphatidyl choline	55	55
Phosphatidyl ethanolamine	26	19
Phosphatidyl serine and inositol	14.5	10
Sphingomyelin	3	6
Phosphatidic acid and cardiolipid	1.5	—

et al.[41]. Thin-layer chromatography for neutral lipids showed the predominant lipid to be phospholipid with progressively lesser amounts of free cholesterol, free fatty acids, triglyceride and cholesterol esters. In Table IV the results are compared to those of total microsomes[42]. Although the phospholipid protein ratio in the Sephadex G-200 is twice that in microsomes, the individual phospholipid composition is virtually identical.

Polyacrylamide gel electrophoresis was performed by the technique of BERKMAN *et al.*[44]. There were fewer protein bands in the purified preparation than in the particulate and dialyzed particulate fractions and two were prominent, but protein was retained at the origin of both spacer and resolving gels (Fig. 5).

Nucleoside diphosphatase activity was found in the G-200 eluate when IDP or ATP were substrates, but not with AMP or CMP. Glucose-6-phosphatase activity was not present.

Since the purified enzyme preparation contained all of the phospholipids present in liver microsomes, the action of fat solvents on enzymatic activity was studied. Using the technique of TABOR[45], an acetone powder was prepared from guinea pig liver. This powder, after suspension in 0.14 M KCl, 0.01 M Tris–HCl (pH 9.0) had enzymatic activity which was retained after dialysis overnight against 0.01 M EDTA, 0.2 mM mercaptoethanol (pH 9.0). Ultrasonic oscillation of this preparation caused

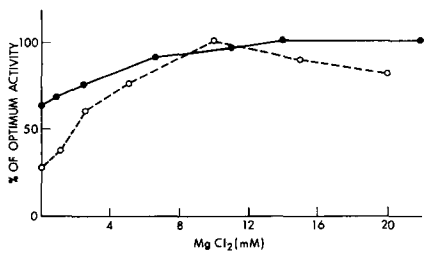


Fig. 6. Effect of Mg^{2+} on UDP-glucuronyltransferase activity in Sephadex G-200 ealute. Enzymatic activity expressed as percentage of optimum observed activity was determined with *o*-aminophenol (●) and bilirubin (○) as substrates in the presence of various concentrations of $MgCl_2$.

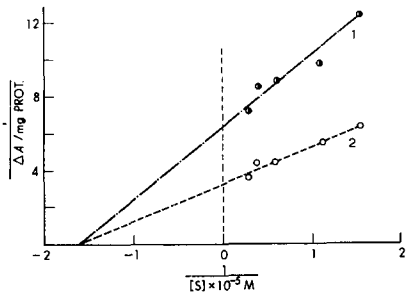


Fig. 7. Lineweaver–Burk plot showing bilirubin conjugation in assays containing no potassium anthranilate (1) and in assays in which its concentration was 1.8 mM (2).

80% loss of enzymatic activity but no solubilization. An acetone–methanol–ether powder similarly prepared had no glucuronidating ability. Enzymatic activity was not restored when the previously extracted lipid, suspended in 0.14 M KCl, 0.01 M Tris (pH 8.0), was simply added to a complete assay system, containing the acetone–methanol–ether powder. The lipid extract, similarly suspended but ultrasonicated or solubilized by the method of FLEISCHER AND KLOUWEN[46] before addition to an assay containing the powder, also failed to restore enzymatic activity. Preincubation at 37° of the powder in 0.14 M KCl and 0.01 M Tris (pH 8.0) and its lipid extract prepared in these various ways before addition to an assay was unhelpful.

Kinetic studies

The influence of inorganic ions on the ability of the Sephadex G-200 eluate to form glucuronides with *o*-aminophenol, *p*-nitrophenol and bilirubin was studied. As shown in Fig. 6, maximal conjugation with *o*-aminophenol and bilirubin required 10 mM $MgCl_2$. In contrast, *p*-nitrophenol conjugation was unaffected by concentrations up to 32 mM $MgCl_2$. With the same enzyme preparation, 6.6 mM $CaCl_2$ did not change *o*-aminophenol conjugation, inhibited bilirubin conjugation by 50% and increased *p*-nitrophenol conjugation by 35%.

As indicated in Table V, the study of K_m values gave variable results even with partially purified preparations. Mean v_{max} (± S.D.) expressed as nmoles glucuronide formed per mg protein for the total microsomal suspension was 92 ± 40 with *o*-aminophenol and 4.2 ± 1.6 for bilirubin, and in the Sephadex G-200 eluate, 294 ± 120 and 24 ± 6 for the same substrates.

TABLE V

UDP-GLUCURONYLTRANSFERASE K_m VALUES

K_m values were determined graphically[47]. Substrates: bilirubin, 1 μmole/mg protein; *o*-aminophenol, 0.1 mmole/mg protein. Results are expressed as means ± S.D. The number of studies with each preparation is given in parentheses.

Enzyme preparation	K_m	
	Bilirubin	*o-Aminophenol*
Total particulate suspension	3.2 ± 1.58 (4)	2.2 ± 1.4 (5)
Sephadex G-200 eluate	5.57 ± 1.3 (7)	2.9 ± 1.14 (6)

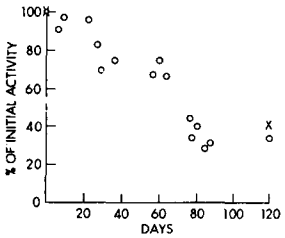


Fig. 8. Stability of UDP-glucuronyltransferase in Sephadex G-200 eluate prepared from livers of hypophysectomized rats. Using bilirubin (○) as substrate enzymatic activity expressed as percentage of initial activity in a preparation stored at 4° is shown. × indicates enzymatic activity (on days 1 and 120), similarly expressed, in the same preparation, in a standard *o*-aminophenol assay to which diethylnitrosamine was added[48].

Potassium anthranilate, 1.83 mM, inhibited bilirubin conjugation but did not interfere with the measurement of bilirubin glucuronide. As shown in Fig. 7, inhibition was noncompetitive. In similar studies, *p*-nitrophenol inhibited *o*-aminophenol glucuronide formation noncompetitively.

Application of purification procedure to livers of animals with diminished conjugating ability

Table VI summarizes the results of these studies. Hypophysectomized or thyroidectomized Sprague–Dawley rats had increased bilirubin-conjugating ability

TABLE VI

PURIFICATION OF HEPATIC GLUCURONYLTRANSFERASE FROM ANIMALS WITH UNUSUAL GLUCURONIDATING ABILITIES

The purification procedure described for guinea pig liver was used without modification. Results are expressed nmoles glucuronide formed per mg protein during the assay.

Substrate	*Animal*	*Enzyme preparation*			
		25% homogenate	*Total particulate fraction*	*Dialyzed particulate fraction*	*Sephadex G-200 eluate*
o-Aminophenol	Normal Wistar rat	3	11	17	65
	Gunn rat	0.2	0.5	1.0	3.0
	Normal Sprague–Dawley rat	1.5	9.0	23	27
	Hypophysectomized Sprague–Dawley rat	0.6	0.6	1.0	3.0
	Thyroidectomized Sprague–Dawley rat	0.5	0.8	3.5	4.0
	Cat	0	—	0	0
Bilirubin	Normal Wistar rat	3	13	23	20
	Gunn rat	0	0	0	0
	Normal Sprague–Dawley rat	2.5	7	11	23
	Hypophysectomized Sprague–Dawley rat	3.0	7	29	40
	Thyroidectomized Sprague–Dawley rat	2.6	7	40	27
	Cat	2.5	—	7.5	16
p-Nitrophenol	Cat	4.6	—	6.5	52

while *o*-aminophenol conjugation was reduced. The addition of diethylnitrosamine (8 mM) to the incubation media restored enzymatic activity with *o*-aminophenol as substrate to levels comparable with that found in normal rats[48].

The stability of UDP-glucuronyltransferase activity in preparations from hypophysectomized rats using bilirubin as the substrate is illustrated in Fig. 8. Assay of *o*-aminophenol glucuronide formation in the presence of 8 mM diethylnitrosamine showed that 40% of the enzymatic activity originally present in the dialyzed particulate fraction and in the Sephadex G-200 eluate, stored at 4°, was present 120 days later[48].

DISCUSSION

The purification achieved by the procedure described is comparable to that of ISSELBACHER *et al.*[13] using snake venom solubilized material, but the stability of enzymatic activity in the Sephadex G-200 eluate was substantially greater in preparations from livers of guinea pig and much more so in those from hypophysectomized rats. In both of these preparations enzymatic activity decreased on storage to a strikingly similar extent with either *o*-aminophenol or bilirubin as substrate.

Although the membranous structures seen on electron microscopy do not necessarily represent structures, *e.g.* endoplasmic reticulum membranes, as they exist in the intact cell[49], the presence of nucleoside phosphatase activity in the enzymatically active Sephadex G-200 eluate and the similarity of its phospholipid content to that of total microsomes together suggest that the enzyme is part of a macromolecular structure derived from the particulate fraction. In addition, the enzyme is associated with other unidentified proteins and is almost certainly not in true solution, *i.e.* "completely surrounded by water molecules"[50]. If gravitational criteria of solubility are satisfied, determination of the lipid content and electron microscopy of the supernatant is advisable before concluding that a membrane-bound enzyme has been made soluble.

Guinea pig liver was chosen as the source of UDP-glucuronyltransferase in this study, since UDP-glucuronic acid is relatively stable in broken cell preparations from this tissue[51], facilitating its use in kinetic studies without the addition of large amounts of UDP-glucuronic acid. Previous investigations have shown that the Mg^{2+} and Ca^{2+} requirements for glucuronide conjugation of *o*-aminophenol[1,52] bilirubin[12,15,24,53] and *p*-nitrophenol[13–15] differ, as found in this study. These differences have been interpreted as indicating that there are multiple forms of UDP-glucuronyltransferase which differ in their inorganic ion requirements, but this conclusion assumes that these ions act directly on enzymatic catalysis. In view of the demonstrated impurity of the enzymatically active material in the Sephadex G-200 eluate, a more likely explanation of the varied and often conflicting results reported is that these ions influence effective donor, acceptor or product concentrations to an extent dependent on nonenzymatic material present in the incubation mixtures. For similar reasons our failure to demonstrate competitive inhibition of bilirubin and *o*-aminophenol glucuronide formation by anthranilic acid and *p*-nitrophenol, respectively, does not necessarily indicate that there are two or more enzymes for the conjugation of glucuronic acid with carboxyl or hydroxyl acceptors. K_m studies undertaken in this investigation to determine whether there was a striking change in K_m during purification suggested that bilirubin

K_m increased during purification, but the results were too inconsistent to have confidence in this conclusion. Such studies with the enzyme preparations available to date do not constitute valid evidence for multiplicity of UDP-glucuronyltransferase. The influence of nonenzymatic contaminants, rather than multiplicity of the enzyme, may also be responsible for the variations in the time of appearance and the rate of increase of the ability to conjugate different substrates in fetal and newborn animals, since in most studies liver homogenates have been used.

Enzymatic activity in liver preparations from species which form glucuronic acid conjugates with some substrates normally but with others poorly or not at all, *e.g.* Gunn rat[11,54–57], cat[10] and hypophysectomized and thyroidectomized rats[58], studied in the stages of the purification procedure devised for guinea pig liver, failed to show any change in the relative ability to conjugate the various substrates tested nor was any substrate conjugated by the purified preparations if it had not been conjugated by the crude homogenate. While these observations do suggest multiplicity of UDP-glucuronyltransferase, confidence in this conclusion is weakened by the observation that diethylnitrosamine added *in vitro* increased the limited ability of hypophysectomized and thyroidectomized rats to conjugate *o*-aminophenol with glucuronic acid to the levels found in normal rats[48], an effect similar to that reported in Gunn rat liver preparations[59].

Our studies leave the hypothesis of multiplicity of UDP-glucuronyltransferase unresolved. We interpret our inability to separate the conjugating capacity for any of the substrates tested and the strikingly similar stability of enzymatic activity with *o*-aminophenol and bilirubin as substrates, in the purified preparations from both guinea pig and hypophysectomized rat, as indicating that if there are indeed multiple forms of the enzyme they have structural features in common which do not permit their separation by the techniques used and which make their rates of degradation similar. Only the preparation of a stable purified enzyme, in true solution and free from contaminants, and its detailed study can ultimately solve the problem.

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