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Author(s)	横田, 博
Citation	大阪大学, 1987, 博士論文
Version Type	VoR
URL	https://hdl.handle.net/11094/1584
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PURIFICATION AND PROPERTIES OF HEPATIC MICROSOMAL DP-GLUCURONYLTRANSEFERASE FROM 3-METHYLCHOLANTHRENE-

HIROSHI YOKOTA

1986

PURIFICATION AND PROPERTIES OF HEPATIC MICROSOMAL UDP-GLUCURONYLTRANSFERASE FROM 3-METHYLCHOLANTHRENE-TREATED RAT

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ABBREVIATIONS

Con A	:	concanavalin A
DTT	:	dithiothreitol
EDTA	:	ethylenediamine tetraacetic acid
Endo H	:	endo-β-N-acetylglucosaminidase H
γ-GTP	:	γ-glutamyl transpeptidase
OVA	:	ovalbumin
SDS	:	sodium dodecyl sulphate
WGA	:	wheat germ agglutinin

PART I

PURIFICATION AND SOME PROPERTIES OF UDP-GLUCURONYLTRANSFERASE FROM 3-METHYLCHOLANTHRENE-TREATED RAT LIVER MICROSOMES

SUMMARY

Hepatic microsomal UDP-glucuronyltransferase has been purified from 3-methylcholanthrene-treated rat by a simple method using DEAE-TOYOPEARL chromatography at room temperature and UDP-hexanolamine-Sepharose chromatography. The purified preparation was homogeneous judging from the results of SDS-polyacrylamide slab gel electrophoresis (54,000 daltons). The purified enzyme catalyzed the glucuronidation of 4-nitrophenol, 1-naphthol and eugenol but did not catalyze the glucuronidation of 4-hydroxybiphenyl, testosterone and bilirubin. This purified isozyme of the transferase could conjugate not only such phenolic xenobiotic substrates as described above but also an endogenous substrate, 5-hydroxytryptamine with high activity. After removing the detergent Emulgen 911 from the purified enzyme preparation, the effects of various phospholipids, detergents and substrate concentration on the the transferase activity were examined. The purified transferase was most activated by lysophosphatidylcholine and phosphatidylcholine among the various phospholipids tested. The activity of the transferase in the presence of phospholipids (toward 4-nitrophenol) was inhibited reversibly by non-ionic detergents and by the addition of an excess amount of the aglycone substrate, 4-nitrophenol. These inhibitors acted on the reconstituted enzyme independently. On the other hand, an excess of the donor substrate, UDP-glucuronic acid, did not inhibit the activity of the enzyme.

INTRODUCTION

Glucuronidation, catalyzed by UDP-glucuronyltransferase(s) [EC 2.4.1.17], is a major conjugation reaction in the biotransformation of a large variety of xenobiotics as well as a number of endogenous compounds in the animal organism (1). In this reaction the substrate is converted to a more water-soluble and less toxic product (1). Several laboratories have so far reported purification of UDP-glucuronyltransferase from liver microsomes of several animal species (2-8). These studies have shown that UDP-glucuronyltransferase represents a family of isozymes that can be classified into three groups according to their substrate specificities, i.e. a) those acting mainly on phenolic substrates (2-5), b) those acting on bilirubin (6), and c) those acting on steroid substrates (7,8).

Although the occurrence of multiple forms of UDP-glucuronyltransferase in rat liver microsomes has been established (9), little is as yet known of the significance of the multiplicity. A marked progress towards this problem is a recent finding by Mackenzie (10) that the cDNA encoding a phenobarbital-inducible form of rat liver UDP-glucuronyltransferase has been isolated, sequenced, and expressed to yield a catalytically active enzyme. To clarify the significance of the multiplicity and inducibility of UDP-glucuronyltransferase, it is necessary to investigate the structure and regulation of UDP-glucuronyltransferase genes and further analysis of the molecular properties of UDP-glucuronyltransferase protein. I report in this paper a simple method for purification of an UDP-

glucuronyltransferase isozyme from liver microsomes of 3-methylcholanthrene-treated rats and some properties of the purified transferase preparation.

MATERIALS AND METHODS

Materials

Cholic acid, purchased from Nissui Yakuhin Co., was further purified and converted to the sodium salt as described (11). Phosphatidylcholine was purified from egg yolk by the method of Singleton et al. (12). Emulgen 911, a nonionic detergent, was supplied by Kao-Atlas Co. DEAE-TOYOPEARL was obtained from Toyo Soda Manufacturing Co. UDP-hexanolamine,3-methyl-cholanthrene, L-α-phosphatidylcholine (Type III-E), L-α-lysophosphatidylcholine (Type I), L-α-phosphatidylethanolamine (Type III), L-α-lysophosphatidylethanolamine, L-α-phosphatidylserine, L-α-lysophosphatidylserine, and bilirubin were purchased from Sigma Chemical Co. [^{14}C]Testosterone (50-60 mCi/mmol) was obtained from Amersham International.

Preparation of UDP-hexanolamine Sepharose 4B --- CNBr-activated Sepharose 4B (10 g) was suspended in 6 ml of 0.1 M bicarbonate buffer (pH 9.5) containing 100 mg of UDP-hexanolamine. The suspension was stirred at 4°C for 36 h and then treated with 0.5 M ethanolamine in 0.1 M bicarbonate buffer (pH 8.0) for 3 h to block residual active sites on the Sepharose. The Sepharose was washed successively with 500 ml of 0.1 M bicarbonate buffer (pH 8.0), 200 ml of 0.1 M potassium phosphate buffer (pH 6.5), and 500 ml of 0.1M potassium phosphate buffer (pH 7.4).

Treatment of Rats --- 3-Methylcholanthrene, dissolved in olive oil, was injected i.p. to male Wistar rats, weighing 250-300 g, at a dose of 80 mg/kg body weight and the animals were

killed 3 days after the injection.

Purification of UDP-Glucuronyltransferase --- All manipulations, except for DEAE-TOYOPEARL chromatography, were conducted at 0-4°C. Liver microsomes were prepared from 3-methycholanthrene-treated rats as described by Imai and Sato (11). The microsomes (200 mg protein) were suspended, to a protein concentration of about 4 mg/ml, in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.6% sodium cholate and 1 mM dithiothreitol (DTT). After stirring for 30 min, the suspension was centrifuged at 105,000 x g for 120 min, and the resultant supernatant fluid was dialyzed overnight against 5 mM potassium-phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 1 mM DTT, 0.2% Emulgen 911, 0.5% sodium cholate, and 20% (v/v) glycerol ("Buffer A "). The dialyzed solution was applied to a column containing the same volume of DEAE-TOYOPEARL that had been equilibrated with Buffer A and maintained at room temperature (13,14). The subsequent washing and elution were also conducted at room temperature. After washing the column with 0.4 column volume of Buffer A, elution was carried out with a 0-0.25 M NaCl gradient in Buffer A. UDP-Glucuronyltransferase activity toward 1-naphthol was thereby eluted at a NaCl concentration of about 0.05 M. Fractions containing high UDP-glucuronyltransferase activities (Fractions 14 through 18 in Fig. 1) were pooled and dialyzed overnight against 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM DTT, 0.05% Emulgen 911, and 20% (v/v) glycerol ("Buffer B"). $MgCl_2$ was added to the dialyzed solution to a concetration of 5 mM and the mixture was applied to a UDP-hexanolamine Sepharose 4B column (0.7 x 10.5 cm) equilibrated

with Buffer B. The column was then washed successively with 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM DTT, 0.2% Emulgen 911, 50 mM NaCl, and 20% (v/v) glycerol ("Buffer C "), and 2 column volumes of Buffer B. The UDP-glucuronyltransferase activity was then eluted with Buffer B containing 10 mM UDP-glucuronic acid and used as the purified enzyme preparation. The Emulgen 911 and UDP-glucuronic acid present in this preparation were removed by washing the preparation on an Amicon Centricon-30 with 0.5 M potassium phosphate buffer (pH 7.4) containing 0.1 mM DTT until ultraviolet absorption due to Emulgen 911 and UDP-glucuronic acid disappeared in the filtrate.

Assay and Other Methods --- UDP-glucuronyltransferase activities toward various substrates were assayed by the methods described in the respective references at aglycone substrate concentrations listed: 1-naphthol (0.5 mM) (15); 4-nitrophenol (0.5 mM) (16); eugenol (0.3 mM) (17); 4-hydroxybiphenyl (0.5 mM) (18); bilirubin (0.1 mM) (19); 5-hydroxytryptamine (1 mM) (20); and testosterone (0.1 mM) (21). One unit of the enzyme was defined as an amount catalyzing the formation of 1 nmol of glucuronide per min. The activities of the purified preparation were assayed in the presence of egg yolk phosphatidylcholine (0.25 mg/ml). Protein concentration was determined by the method of Lowry et al. (22) using bovine serum albumin as a standard. Sodium dodecylsulfate (SDS)-polyacrylamide slab gel electrophoresis was performed as described by Laemmli (23) using 10% gel and the gels were stained with Coomassie brilliant blue. Phospholipids were dried and suspended in 50 mM Tris-HCl buffer (pH 7.0) to a concentration of 2 mg/ml and the suspension was sonicated for 10

min. The sonicated suspension was centrifuged at 10,000 x g for 30 min and the supernatant fluid was used as activators of the purified UDP-glucuronyltransferase.

RESULTS

Purification

An isozyme of UDP-glucuronyltransferase that conjugates 1-naphthol actively was purified from liver microsomes of 3-methylcholanthrene-treated rats as described under "MATERIALS AND METHODS". The purification method employed, consisting of solubilization, DEAE-TOYOPEARL chromatography, and UDP-hexanolamine Sepharose chromatography steps, is simple and can be completed within 3 days. DEAE-TOYOPEARL chromatography was conducted at room temperature to improve separation efficiency (13,14). Incubation of the enzyme preparation at room temperature for 10 h in the buffer used for the chromatography (Buffer A) resulted in only a slight loss of the activity (5-10% at most). As shown in Fig. 1, in this chromatography step protein was eluted in two major peaks. The UDP-glucuronyltransferase activity was largely associated with the first peak, whereas cytochrome P-450, the major protein component of the microsomes, was recovered in the second peak. It was thus possible to obtain about 45% of the UDP-glucuronyltransferase activity applied in a state free from cytochrome P-450. When the UDP-glucuronyltransferase preparation thus obtained was dialyzed and subjected to UDP-hexanolamine Sepharose chromatography, about 75% of the charged protein passed through and about 90% of the UDP-glucuronyltransferase activity was adsorbed to the column (Fig. 2). After elution of a small amount of nonspecifically adsorbed protein with a buffer containing 50 mM NaCl (Buffer C), the UDP-glucuronyltransferase still tightly bound by the column could be

eluted as a sharp band with Buffer A containing 10 mM UDP-glucuronic acid. As shown in Table I, this purification method resulted in an approximately 40-fold purification of UDP-glucuronyltransferase activity toward 1-naphthol from the solubilized microsomes with an overall yield of 17%. It should be noted, however, that the specific activity determined here (about 4,000 units/mg protein) is an underestimated value, because this determination was made in the presence of detergents which inhibit the UDP-glucuronyltransferase activity, as described below. The final preparation gave a single protein band on sodium dodecylsulfate-polyacrylamide slab gel electrophoresis (Fig.3) and an apparent molecular weight of 54,000 was determined for the protein band.

Properties

As shown in Table II, the UDP-glucuronyltransferase isozyme purified in this study catalyzed actively the conjugation of 4-nitrophenol, 1-naphthol, and eugenol, but its activities toward 4-hydroxybiphenyl and testosterone were very low. It was completely devoid of bilirubin conjugating activity. A notable finding was that 5-hydroxytryptamine, an endogenous compound, was as actively conjugated as eugenol, a xenobiotic substrate, by the enzyme. That 5-hydroxytryptamine was conjugated by the same UDP-glucuronyltransferase as that catalyzing the conjugation of xenobiotic substrates could be confirmed by the findings that 5-hydroxytryptamine conjugation was inhibited by 4-nitrophenol (Fig. 4A) and 4-nitrophenol conjugation was inhibited by 5-hydroxytryptamine (Fig. 4B). The data shown in Fig. 4 further suggested that 4-nitrophenol had a higher affinity that 5-

hydroxytryptamine for the enzyme.

The purified enzyme was washed with 0.5 M potassium phosphate buffer (pH 7.4) containing 0.1 mM DTT on a "Centricon-30" (Amicon) until the UV absorbance of UDP-glucuronic acid and Emulgen 911 in the filtrate became undetectable. The specific activity of the purified UDP-glucuronyltransferase preparation containing 0.05% Emulgen 911 was 99.5 units per mg of protein, whereas that of the washed enzyme containing no detergents was 443.3 units per mg of protein. Both the detergent-free and -containing enzyme preparations (2 μg) were activated by the addition of lysophosphatidylcholine in the assay medium and the same maximum specific activities were obtained by the addition of 1.5 mg/ml lysophosphatidylcholine (the detergent-free preparation) and 6.0 mg/ml lysophosphatidylcholine (the preparation containing 0.05% Emulgen 911) (Fig. 5). Effects of various phospholipids on the activity of the detergent-free enzyme are shown in Table III. The activity was most activated by lysophosphatidylcholine (11-fold) and egg yolk phosphatidylcholine (9.5-fold) in agreement with a previous work (24) in which a phospholipid-free preparation prepared by ammonium sulfate fractionation from phospholipase A_2-treated liver microsomes was used. The UDP-glucuronyltransferase activity reconstituted with lysophosphatidylcholine was reduced by the addition of Emulgen 911 to the reaction mixture (Fig. 6). The Emulgen 911-induced inhibition in the presence of 1.5 μg of lysophosphatidylcholine was higher than that in the presence of 15 μg of the phospholipid (Fig. 6). The inhibition was released by re-addition of an excess amount of the phospholipid (Fig. 6). These results indi-

cated that the inhibition of UDP-glucuronyltransferase activity by Emulgen 911 was competitively related with the activation process by phospholipids. The effects of various detergents on the purified UDP-glucuronyltransfease activity reconstituted with lysophosphatidylcholine are shown in Table IV. Non-ionic detergents such as Emulgen 911, Triton X-100 and Tween 80 at a concentration of 0.02% decreased the activity of the purified enzyme in the presence of lysophosphatidylcholine and the reduced activities were re-activated by the re-addition of the phospholipid. On the other hand, sodium cholate was not significantly inhibitory on the activity at the concentration examined (Table IV). The effect of substrate concentration on the activity toward 4-nitrophenol is shown in Fig. 7. When 1.0 μg of the enzyme was used, higher concentrations of 4-nitrophenol were rather inhibitory and maximum activities were obtained at an aglycone substrate concentration of about 2 mM for both the detergent-free and -containing preparations. When the enzyme assay was performed using 2.5 μg (2.5-fold) of the purified enzyme, the optimal 4-nitrophenol concentration was shifted to 5 mM (2.5-fold) (Fig. 7A). The activity of the purified enzyme (1 μg of enzyme protein) was not inhibited by high concentrations of the donor substrate, UDP-glucuronic acid (10 mM) (Fig. 7B). Even by 30 mM UDP-glucuronic acid, the activity was not inhibited (data not shown).

DISCUSSION

Since the UDP-glucuronyltransferase activity toward 4-nitrophenol solubilized from liver microsomes of 3-methylcholanthrene-treated rats was stable at room temperature at least for 10 h in 5 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.2% Emulgen 911, and 0.1 mM DTT, DEAE-TOYOPEARL chromatography at room temperature was adopted as the first purification step using the above-mentioned solvent system. The room temperature chromatography on DEAE-resins has been shown to be effective for separation of different isozymes of cytochrome P-450 (13,14). In this study, this method was also found to be effective to achieve separation of different isozymes of UDP-glucuronyltransferase. In fact, the UDP-glucuronyltransferase activity toward 4-nitrophenol was clearly separated from those toward 4-hydroxybiphenyl and bilirubin as well as the major component of the microsomes, cytochrome P-450. Further purification was performed by affinity chromatography on a UDP-hexanolamine Sepharose. In this step it was found that the protein concentration of applied sample should be higher than 0.2 mg/ml in order to achieve a high yield of the enzyme. Although most of the inert proteins passed through the column, a small amount of nonspecifically bound proteins remained on the column in addition to the UDP-glucuronyltransferase . These nonspecifically bound proteins could, however, be removed by washing the column with a buffer containing 50 mM NaCl. The UDP-glucuronyltransferase tightly bound to the affinity column could then be eluted with a buffer containing 10 mM UDP-glucuronic

acid. This purification method consisting of solubilization and two chromatography steps is much simpler than those developed previously (3-5) and also reproducible. By this method about 17% of the UDP-glucuronyltransferase activity toward 4-nitrophenol present in the solubilized supernatant could be recovered in the final preparation.

Purification of several UDP-glucuronyltransferase isozymes which catalyzes the glucuronidation of endogenous and xenobiotics substances from rat liver has been reported in several laboratories (2-4, 6-8). The isozyme isolated in the present study catalyzes the conjugation of 4-nitrophenol, 1-naphthol, eugenol and 5-hydroxytryptamine but is practically inactive toward 4-hydroxybiphenyl, bilirubin and testosterone (Table II). The substrate specificity of this isozyme and its subunit molecular weight estimated from SDS-gel electrophoresis indicate that it is identical or closely similar to the isozyme purified by Burchell (2) from phenobarbital-treated male rats, "GT1" purified by Bock et al. (4) from phenobarbital-treated male rats, and "PNP-GT" purified from 3-methylcholanthrene-treated female rats by Falany and Tephly (5). Although these isozymes are only concerned with glucuronidation of xenobiotic substrates, the UDP-glucuronyltransferase preparation purified in this study was found to catalyze the conjugation of 5-hydroxytryptamine (serotonin), an endogenous compound, in addition to phenolic xenobiotics such as 4-nitrophenol. The data shown in Fig. 4 further suggest that the same catalytic site on the enzyme is involved in the conjugation of 5-hydroxytryptamine and 4-nitrophenol. Therefore, it is likely that one physiological

function of this isozyme *in vivo* is to catalyze the glucuronidation of endogenous substrates including 5-hydroxytryptamine.

The fact that purified UDP-glucuronyltransferase preparations containing dtergents (3, 4) and partial purified preparation (16) required phospholipids for full activities has been reported by several investigators. In this study, the detergent, Emulgen 911, was removed from the enzyme preparation and effects of various phospholipids and detergents on the activities of the enzyme was investigated. The data shown in Fig. 5 indicated that phosphatidylcholine and lysophosphatidylcholine are the best activators in agreement with the findings reported by Singh *et al.*(25). On the other hand, the activity of the purified UDP-glucuronyltransferase reconstituted with lysophosphatidylcholine is inhibited by the addition of detergents and reactivated by re-addition of phospholipids in agreement with Erickson *et al.* (24) who used a delipidated delipidated microsomes from guinea pigs. Erickson *et al.* reported (24) that the extent to which phospholipids reversed the detergent-induced inhibitions was dependent on the type of detergents used. Thus the effect of cholate was reversed completely, whereas inhibition due to Triton X-100 and Tween 80 was more difficult to overcome by addition an excess amount of phospholipids. In the case of the UDP-glucuronyltransferase isozyme purified in this study, cholate (0.02%) could not significantly inhibit the UDP-glucuronyltransferase activity in the presence of phospholipid, but those caused by Emulgen 911, Triton X-100 and Tween 80 were reversed by adding an excess amount of phospholipids (Table 4). These results suggest that non-ionic detergents bind to the

hydrophobic sites of the purified UDP-glucuronyltransferase and/or the phospholipid, therby interferring with the interaction between the UDP-glucuronyltransferase molecule and phospholipids, we have noticed that partially purified UDP-glucuronyltransferase preparations containing 0.05-0.2% Emulgen 911 could not bind to DEAE-cellulose and CM-cellulose at neutral pH. The UDP-glucuronyltransferase protein might be deeply buried in large micelles of non-ionic detergents and phospholipids. The inhibition by these non-ionic detergents might be concerned with that. The inhibition of the purified UDP-glucuronyltransferase by excess addition of aglycone substrate was found (Fig. 7A). On the other hand, the activity was not inhibited by excess addition of the donor substrate, UDP-glucuronic acid (Fig. 7B). The optimal concentration of the aglycone substrate was dependent on the amount of the enzyme protein. The mechanism of this xenobiotic substrate inhibition is not clear, but this inhibition would be one of interesting characters of the UDP-glucuronyltransferase , because it might be concerned with regulation or induction of the enzyme by administration of xenobiotic substrates to animals (26).

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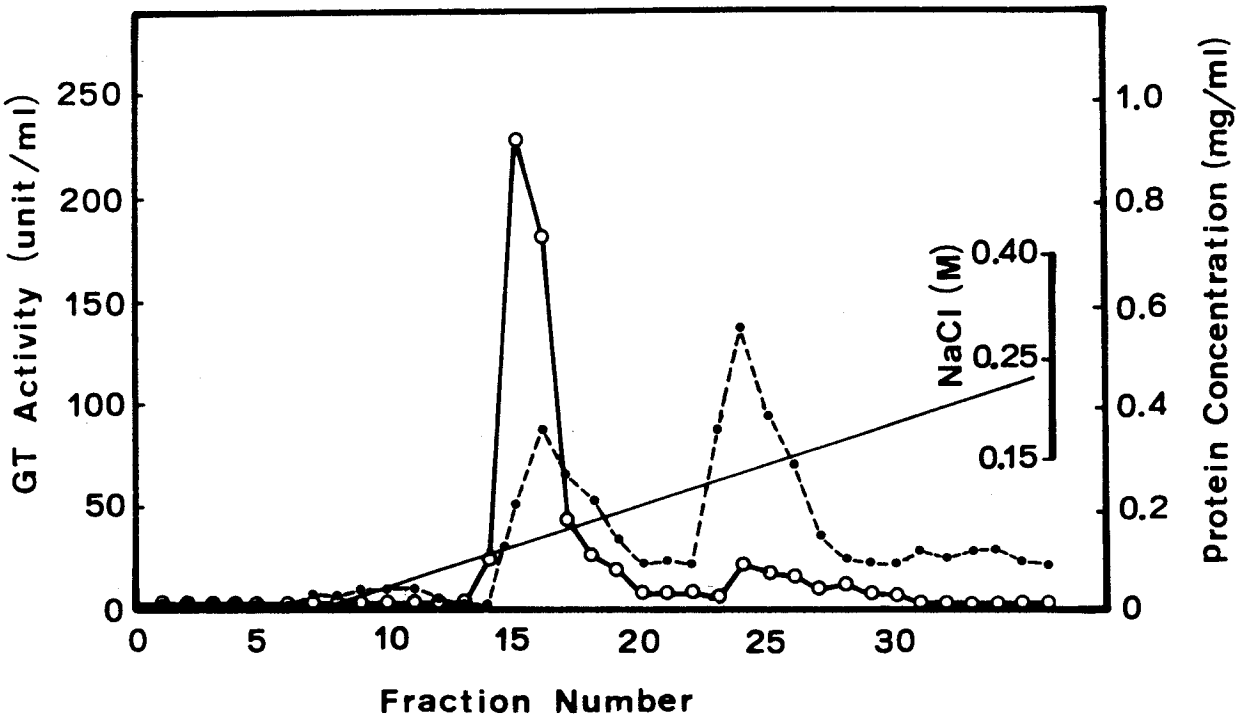

Fig. 1 Elution profile of UDP-glucuronyltransferase on DEAE-TOYOPEARL chromatography. (●): Enzyme activity toward 1-naphthol, (○): protein concentration. The column (2.3 x 12.0 cm) was equilibrated with 5 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 0.5% sodium cholate, and 0.2% Emulgen 911. A linear gradient of NaCl was used (0 to 0.25 M) in the same buffer mixture. The entire chromatographic procedure was performed at room temperature.

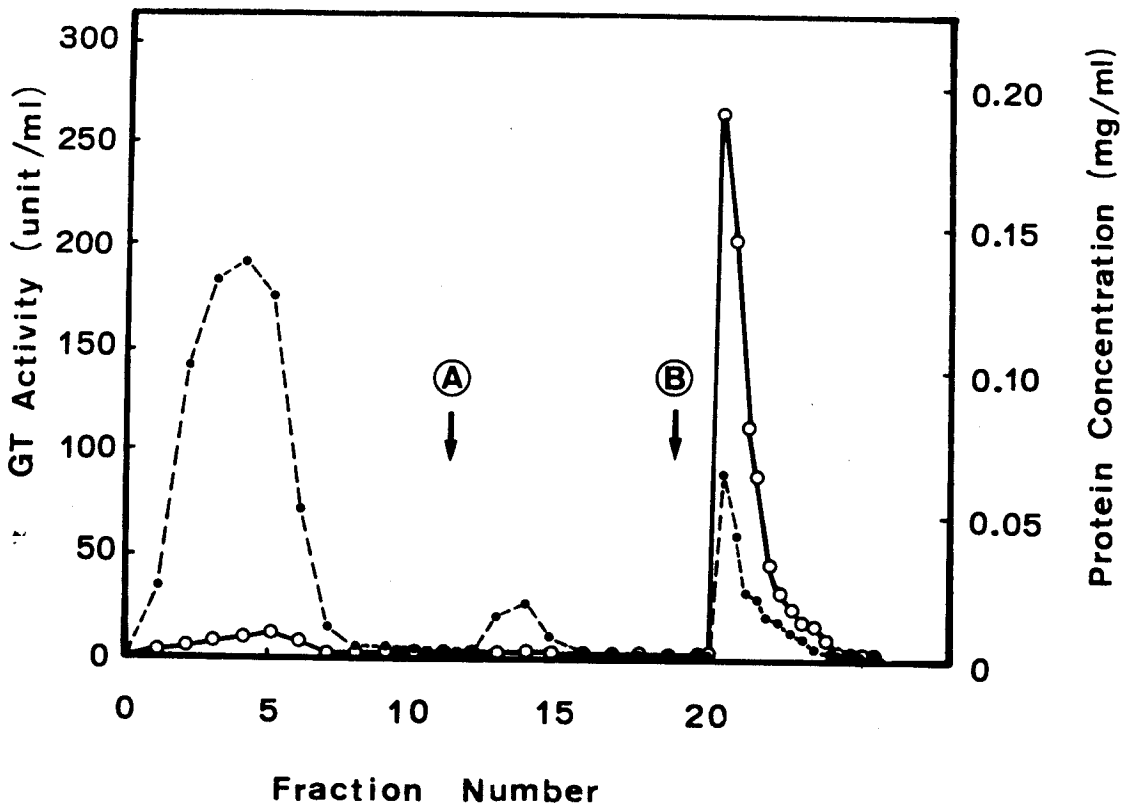

Fig. 2 Elution profile of UDP-glucuronyltransferase on UDP-hexanolamine Sepharose 4B chromatography. (○): Enzyme activity toward 1-naphthol, (●): protein concentration. The active fraction (approx. 2.9 mg of protein) from DEAE-TOYOPEARL chromatography was applied to a column (0.7 x 10.5 cm) equilibrated with 10 mM Tris-HCl (pH 8.0) containing 20% glycerol, 0.05% Emulgen 911, and 0.1 mM DTT ("Buffer B"). The column was washed with 5 column volumes of 10 mM Tris-HCl buffer (pH 8.0), containing 20% glycerol, 50 mM NaCl, 0.2% Emulgen 911, and 0.1 mM DTT at arrow Ⓐ. Enzyme was eluted with "Buffer B" containing 10 mM UDP-glucuronic acid at arrow Ⓑ. The enzyme activities of these fractions were determined after the addition of egg yolk phosphatidylcholine (about 50 μg) to the assay mixture.

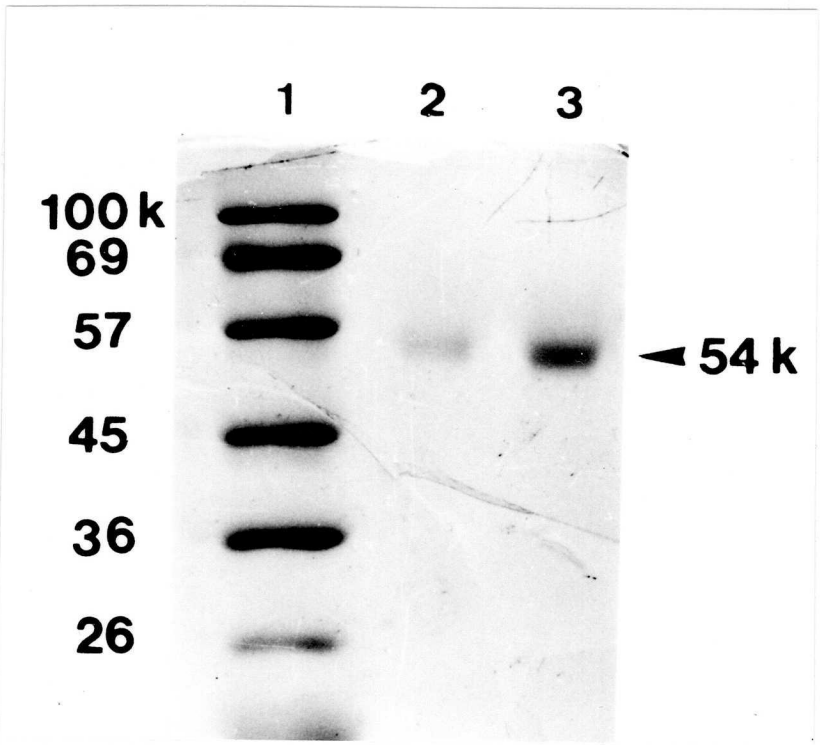

Fig. 3 SDS-polyacrylamide slab gel electrophoresis of UDP-glucuronyltransferase purified from 3-methylcholanthrene-treated rat liver. Electrophoresis was carried out as described in "MATERIALS AND METHODS". Lane 1: marker proteins of molecular weight (phosphorylase b, bovine serum albumin, pyruvate kinase, ovalbumin, lactate dehydrogenase, chymotrypsinogen A, and cytochrome c), lanes 2 and 3: 0.5 and 5.0 μg of purified UDP-glucuronyltransferase, respectively.

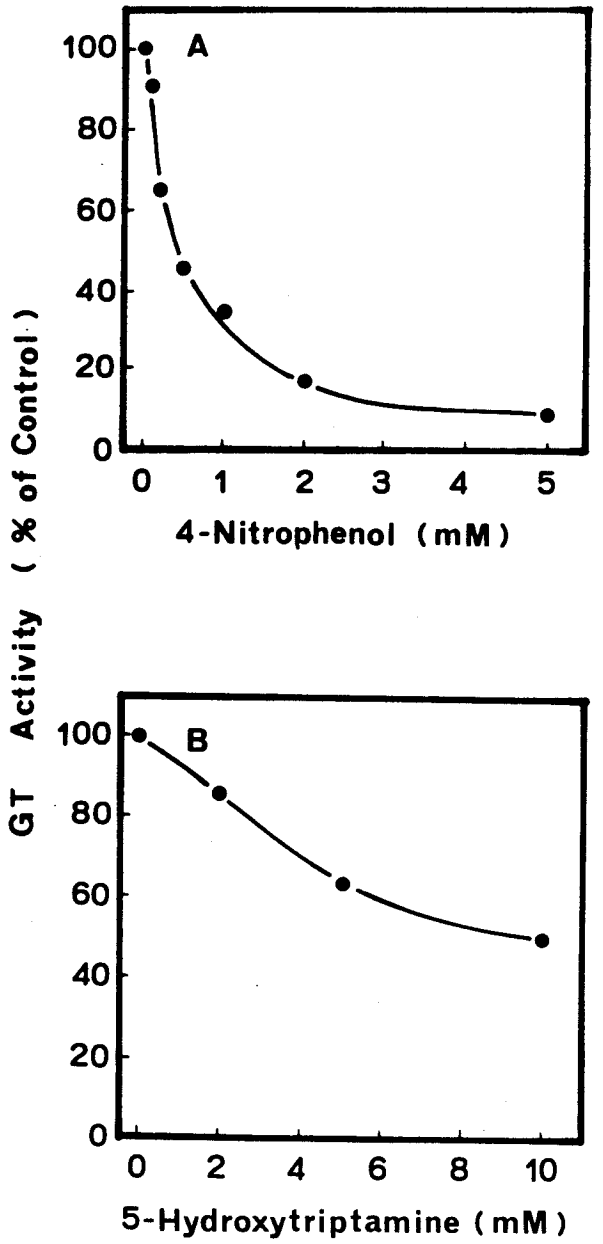

Fig. 4 Inhibition of UDP-glucuronyltransferase by aglycone substrates. The purified enzyme (1.5 μg) was incubated with egg yolk phosphatidylcholine at 50 ug in the assay mixture at 4°C for 30 min, and then the enzymatic activities were assayed toward 5-hydroxytryptamine at various concentrations of 4-nitrophenol (A) and toward 4-nitrophenol at various concentrations of 5-hydroxytryptamine (B).

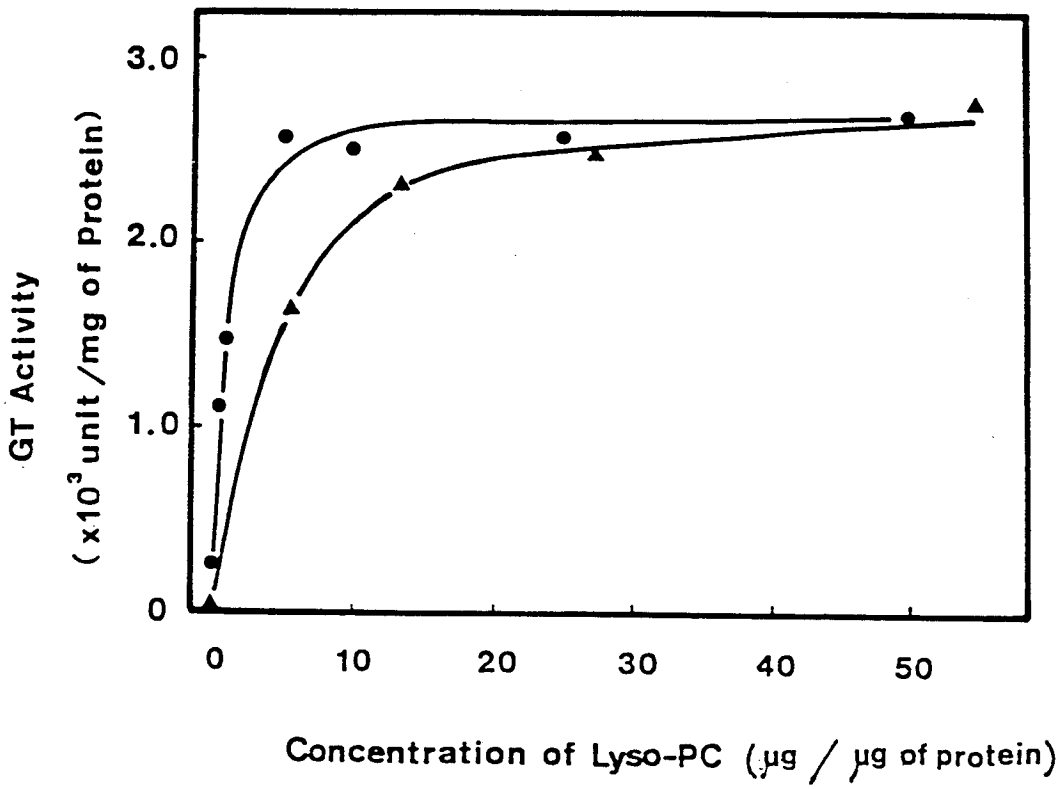

Fig. 5 Effect of lysophosphatidylcholine on the purified UDP-glucuronyltransferase activity toward 4-nitrophenol. Detergent-free enzyme was prepared and assayed (● : 2.0 μg) by the method described in "MATERIALS AND METHODS". The enzyme (▲ : 2.0 μg) containing 0.05% Emulgen 911 had been preincubated with various concentrations of lysophosphatidylcholine at 4°C for 30 min before the activity was assayed.

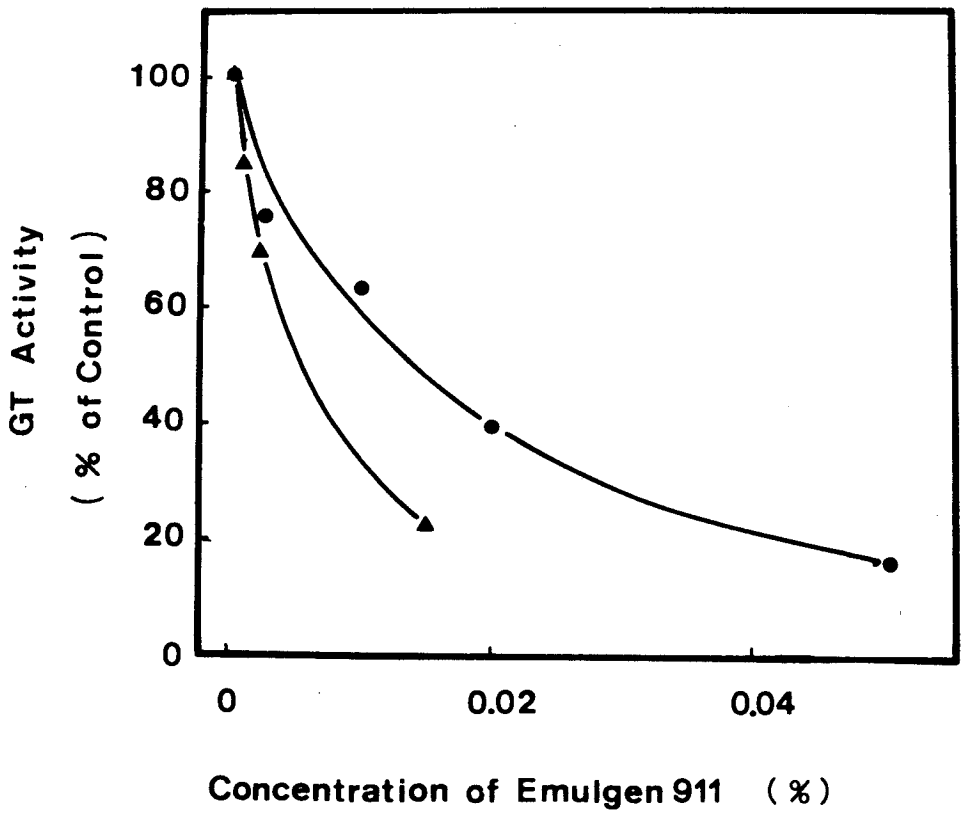

Fig. 6 Effect of Emulgen 911 on UDP-glucuronyltransferase activity toward 4-nitrophenol. The purified enzyme (1.5 μg) was incubated with 1.5 μg (▲) or 15.0 μg (●) of lysophosphatidylcholine at 4°C for 30 min and then assayed in the presence of various concentrations of Emulgen 911. Specific activities of the detergent-free enzyme corresponded to 977 and 2,952 units per mg of protein in the presence of 1.5 and 15.0 μg of lysophosphatidylcholine, respectively.

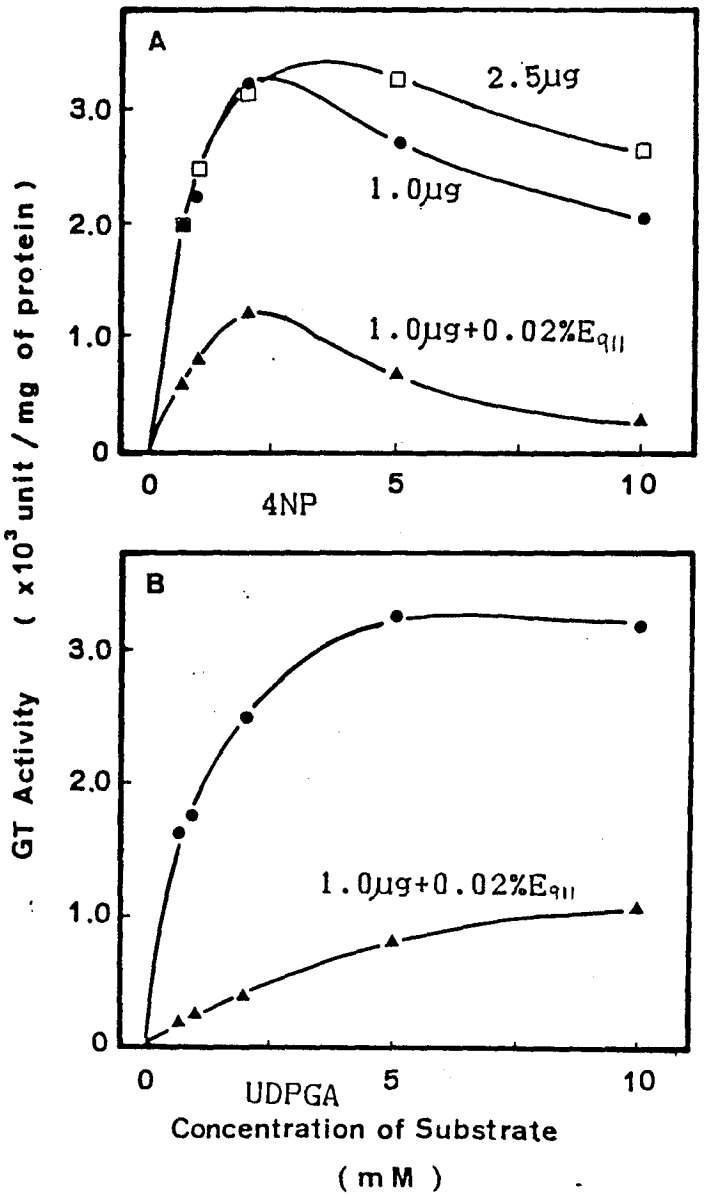

Fig. 7 Effects of 4-nitrophenol and UDP-glucuronic acid on UDP-glucuronyltransferase activity toward 4-nitrophenol. The detergent-free purified UDP-glucuronyltransferase was preincubated with lysophosphatidylcholine (10 μg per μg of protein) at 4°C for 30 min. Activities of the enzyme toward 4-nitrophenol were assayed in the absence (● : 1.0 μg of protein, □ : 2.5 μg of protein) and in the presence (▲ : 1.0 μg of protein) of 0.02% Emulgen 911. Effect of the concentration of 4-nitrophenol (glucuronate acceptor) on UDP-glucuronyltransferase activity in the presence of 5 mM UDP-glucuronic acid (A). Effect of the concentration of glucuronic axid (glucuronate donor) on the enzymatic activity in the presence of 2 mM 4-nitrophenol (B).

Table I. Purification of 1-naphthol UDP-glucuronyltransferase from 3-methylcholanthrene-treated rat liver microsomes.

Purification step	Total protein (mg)	Specific activity (unit/mg) [a]	Total activity (unit)	Yield (%)
Solubilized microsomes	180.0	111.5	20070.0	100
DEAE-TOYOPEARL chromatography	12.0	746.9	8962.8	45
UDP-hexanolamine Sepharose-4B chromatography	0.84	4040.0 [b]	3393.6	17

[a] One unit of activity corresponds to 1 nmol of substrate conjugated per minute.

[b] The specific activity of this enzyme (1.5 μg) was determined after the addition of egg yolk phosphatidylcholine about 50 μg to the assay mixture.

Table II. Aglycone substrate specificity of purified UDP-glucuronyltransferase

Substrate	Specific activity (unit/mg)[a]	Purification
1-Naphthol	4040.0	36
4-Nitrophenol	5017.0	25
Eugenol	1838.0	37
5-Hydroxytryptamine	1669.0	12
4-Hydroxybiphenyl	91.8	2.4
Bilirubin	N.D.	-
Testosterone	5.2	1.7

[a] The specific activity of this enzyme (1.5 µg) was determined after the addition of egg yolk phosphatidylcholine (about 50 µg) to the assay mixture.

Table III. Effects of phospholipids on activity of 4-nitrophenol UDP-glucuronyltransferase. Detergent (Emulgen 911) was removed from this enzyme solution and the enzyme (1.8 μg) was incubated with lipids (40 μg) at 4°C for 30 min before the activity was assayed.

Phospholipid	Activity (unit/mg)	ratio
None	316	1.0
Egg yolk phosphatidylcholine	3006 [a]	9.5
Phosphatidylcholine	1234	3.9
Lysophosphatidylcholine	3607	11.4
Phosphatidylethanolamine	506	1.6
Lysophophatidylethanolamine	918	2.9
Phosphatidylserine	1076	3.4
Lysophosphatidylserine	538	1.7
Sphingomyerine	348	1.1

[a] Phosphatidylcholine (about 50 μg) purified from egg yolk by the method in "Materials and Methods" was added to the assay mixture.

Table IV. Effects of various detergents on the UDP-glucuronyltransferase activity toward 4-nitrophenol. The purified enzyme (1.5 μg) was incubated with lysophosphatidylcholine (1.5 μg or 15.0 μg) at 4°C for 30 min and assayed in the presence of various detergents (0.02 %) in the assay mixture.

Detergents	1.5 μg of PC		15.0 μg of PC	
	Specific activity (unit/mg)	Ratio (%)	Specific activity (unit/mg)	Ratio (%)
None	977	100	2952	100
Emulgen 911	168	17	1131	39
Triton X-100	404	41	1206	41
Tween 80	519	53	1817	62
Cholate	958	98	2601	88
SDS	0	0	0	0

PART II

INDUCTION OF THE UDP-GLUCURONYLTRANSEFERASE

SUMMARY

Antibodies were raised against the UDP-glucuronyltransferase purified in "PART I". The antibodies inhibited the activities of the purified enzyme and those of solubilized liver microsomes to catalyze the conjugation of 1-naphthol, 4-nitrophenol, and 5-hydroxytryptamine. However, the antibodies did not affect on the conjugating activity toward 4-hydroxybiphenyl, which has been reported to be conjugated by a different isozyme of UDP-glucuronyltransferase, and also the activity of NADPH—cytochrome P-450 reductase in the solubilized microsomes. Immunoblotting analysis showed that the antibodies recognized the purified enzyme having a molecular weight of 54,000 daltons and bound to only a single protein among microsomal proteins. From the data of immunoblotting, the level of this isozyme protein in hepatic microsomes was found from the data of immunoblotting to be increased by administration of 3-methylcholanthrene, phenobarbital, and polychlorinated biphenyls and also in microsomes from hyperplastic nodule cells fomed by dietary administration of 0.05% 2-acethylaminofluorene to rats for 6 weeks. It was observed that the level of translatable mRNA encoding this isozyme was increased in liver microsomes of 3-methylcholanthrene-treated rats by *in vitro* translation assays. It was found that this isozyme occurs in microsomes from kidney, lung, and intestine, and the enzyme protein in these organs was also increased by treatments of rats with 3-methylcholanthrene or phenobarbital.

INTRODUCTION

Glucuronidation is a major pathway in detoxification of many metabolites, toxics, and xenobiotics. Hepatic microsomal UDP-glucuronyltransferase represents a family of isozymes which catalyze conjugation of many xenobiotics and endogenous compounds with UDP-glucuronic acid. The resulting glucuronides are generally less biologically active and more polar than aglycone substrates, and they are readily excreted in urine and bile (5). Individual UDP-glucuronyltransferase activites which catalyze the glucuronidation of a limited group of substrates can be specifically induced in mammalian liver by certain compounds such as 3-methylcholanthrene, phenobarbital and polyclorinated biphenyls (PCBs) (6-9). It has been shown that increased UDP-glucuronyltransferase levels observed in 3-methylcholanthrene-treated rat liver, preneoplastic liver nodules, and certain Morris hepatomas are shown to be due to increases in the amount of the enzyme protein in the correspoding microsomes (10,11). Recently, Jackson et al. (12) have reported that phenobarbital treatment of chick embryo resulted in a large increase in UDP-glucuronyltransferase activity in the liver and that this increase can be accounted for by an immunochemically quantifiable increase of a single isozyme of UDP-glucuronyltransferase protein and by an enhancement of the corresponding translatable UDP-glucuronyltransferase mRNA. In this paper, we show that the induction of UDP-glucuronyltransferase activities can be attributed to be increase of the UDP-glucuronyltransferase isozyme protein not only in liver microsomes but also in kidney, lung, and intes-

tine microsomes, and this induction is corelated with an increment of mRNA encoding the isozyme also in 3-methylcholanthrene-treated rat liver microsomes.

MATERIALS AND METHODS

Materials --- Cholic acid purchased from Nissui Yakuhin Co. was futher purified and converted to the sodium salt as described previously (13). Phenobarbital and 3-methylcholanthrene were purchased from Sigma Chemical Co. A polychlorinated biphenyl (PCB) mixture, Kanechlor 300 (chlorine content, 42%) was generouly supplied by Dr. T. Shimada, of Osaka Prefectural Institute of Public Health. Nitrocellulose sheets were purchased from Toyo Roshi Co., anti-rabbit IgG-peroxidase conjugate was from Jackson Immuno-Research, [^{35}S]methionine (1110 Ci/mmol; 1Ci=37GBq) was from Amersham. [^{14}C]protein A (2.1 mCi/nmol) was a generous gift of Dr. M. Nakamura, Institute for Protein Research, Osaka University. Pure NADPH-cytochrome P-450 reductase and specific antibodies against this enzyme were generous gift of Dr.M.Iwasaki, Institute for Protein Reaearch, Osaka University.

Treatment of Animals --- Male Wister rats (250 g) were used. 3-Methylcholanthrene and polychlorinated biphenyls (PCB) were dissolved in olive oil and injected intraperitoneally at doses of 80 mg/kg of body weight and 500 mg/kg of body weight, respectively, and the rats were killed on the 4th day, and 5th day, respectively. Phenobarbital was dissolved in saline and given to rats by daily intraperitoneal injection of 100 mg/kg of body weight and the rats were killed on 2 days after the final injection. 2-Acetylaminofluorene was dietary administered to the rats. Diet containing 0.05% acetylaminofluorene was fed to the experimental animals for 6 weeks with a week interval and these

rats were killed on 3 weeks after the final administration. Hyperplastic nodules were formed in the 2-acetylaminofluorene-treated rat livers and the nodule cells were collected and used for the following experiments.

Preparation of Microsomes from various organs of rat --- Rat liver, after being perfused with 0.15 M KCl, was minced and homogenated with 4 volumes of the same solution. The homoginate was centrifuged for 15 min at 9,000 x g. Then, the supernatant fraction was further centrifuged at 105,000 x g for 60 min to obtain microsomes. Microsomes from rat kidney, lung, intestine, and brain were also prepared by the method as described above.

Preparation of Antibodies against UDP-Glucuronyltransferase --- UDP-glucuronyltransferase from liver microsomes of 3-methylcholanthrene-treated rats was purified as described (14). The purified enzyme (80 μg) was emulsified with Freund's complete adjuvant and injected into a rabbit (2.0 kg) at multiple intracutaneous sites. Weeks later, three booster doses of 80 μg of the enzyme protein emulsified in the same adjuvant were given at 10 days intervals. The blood of the immunized animals was collected from the ear vein. The antiserum was brought to 50% saturation with saturated ammonium sulfate solution (pH 7.0), and the precipitate was collected by centrifugation and dissolved in 10 mM sodium phosphate buffer (pH 7.5) containing 0.9% NaCl (PBS). The solution was again brought to 45% saturation with saturated ammonium sulfate solution and the precipitate was dissolved in PBS and dialyzed overnight against 300 volumes of the

same buffer.

Inhibition of UDP-Glucuronyltransferase Activities --- Microsomes (5 mg/ml) suspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM DTT were solubilized with 1.0% sodium cholate. The 105,000 x g supernatant was incubated overnight at 4°C with various amounts of anti-UDP-glucuronyltransferase immunoglobulin as indicated in 20 mM potassium phosphate buffer (pH 7.4) containing 0.12% sodium cholate, 0.01% DTT. Enzyme activities in the mixtures were determined.

SDS-Polyacrylamide Slab Gel Electrophoresis and Immunoblotting --- SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli.(15). Proteins were transferred to nitrocellulose sheets essentially by the method of Howe and Hershey (16), except that the transfer buffer contained 348 mM glycine, 50 mM Tris base, and 20% (v/v) methanol. The transfer was carried out at 200 mA (5-6 V) for 5 h. The step of Amido Black staining was omitted. For detecting of the UDP-glucuronyltransferase isozyme band, after reacting with the rabbit anti-rat UDP-glucuronyltransferase immunoglobulin, goat anti-rabbit IgG peroxidase conjugate was applied. Nonspecific binding sites on the nitrocellulose sheets were blocked overnight in 1.0% bovine serum albmin dissolved in 10 mM phosphate buffered saline (PBS), pH 7.5. The blocked sheets were incubated with the primary antibodies (rabbit anti-rat UDP-glucuronyltransferase immunoglobulin) in PBS containing 1.0% bovine serum albumin and 0.1% Triton X-100 (AB buffer) for 3 h at room temperature. The sheets were washed with AB buffer and PBS. The washed sheets were subsequently incubated with the secondary antibodies (goat

anti-rabbit IgG-peroxidase conjugate) diluted 1:1000 in AB buffer for 3 h at room temperature. Immunoblotts were stained using 0.125% diaminobenzidine as a substrate of peroxidase in 0.05 M Tris-HCl (pH 7.6) containing 0.004 % H_2O_2. When the ratio of the UDP-glucuronyltransferase protein and NADPH-cytochrome P-450 reductase was measured, the sheets washed with AB buffer were incubated with ^{14}C-labeled protein A (2.1 mCi/nmol), and the bands were detected by fluorography. Radioactivities of the two bands corresponding to the UDP-glucuronyltransferase and NADPH-cytochrome P-450 reductase on the nitrocellulose sheet were counted.

Analytical Procedure --- UDP-glucuronyltransferase activities toward various substrates were assayed at the following concentrations of aglycone and by the methods described in respective references : 0.5 mM 1-naphthol (17), 0.5 mM 4-nitrophenol (18), 0.5 mM 4-hydroxybiphenyl (19), 1.0 mM 5-hydroxytryptamine (20), 0.1 mM bilirubin (21). Cytochrome P-450 in microsomes was estimated from the CO difference spectrum of dithionite reduced sample as described by Omura and Sato (22). NADPH-cytochrome P-450 reductase was determined by the method of Omura et al. (23). Protein was determined by the method of Lowry et al. (24) using bovine serum albumin as a standard.

Preparation of RNA from Rat Liver --- Total liver RNA was isolated by the guanidine thiocyanate cesium chloride procedure (25). Livers (5 g) were rapidly homogenized in 120 ml of the homogenizing buffer (4 M guanidine thiocyanate, 0.1 M 2-mercaptoethanol, 25 mM sodium citrate, pH 7.0). The homogenate was centrifuged at 25,000 x g for 10 min at 20°C. The super-

natant (2.5 ml) was layered over 5 ml of 5.7 M cesium chloride buffered with 0.1 M EDTA and centrifuged for 20 h at 25,000 rpm and 20°C in a Beckman SRP-28 rotor. The RNA pellet was dissolved in 100 ml of distilled water at 55°C and precipitated at -20° by addition of 10 ml of 3 M sodium acetate and 250 ml absolute ethanol. After one night, the precipitated RNA was centrifuged at 10,000 rpm for 10 min at 4°C. The pellet was washed with 70% ethanol, dissolved in 10 ml of distilled water containing 0.1% SDS at 55°C and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was reprecipitated by the addition of 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ethanol at 20°C. After one night, the final RNA was dissolved in distilled water at a concentration of 20-30 A_{260} units/ml and used in translation assays.

In Vitro Protein Synthesis and Immunoprecipitation --- Total RNA from the livers of untreated and 3-methylcholanthrene-treated rats (final concentration, 5 A_{260} unit/ml) was translated at 28°C for 60 min in a wheat-germ (S-23) extract (total volume, 25ul) containing [^{35}S]methionine (800 uCi/ml), as described (26). After translation, UDP-glucuronyltransferase synthesized was immunoprecipitated with anti-UDP-glucuronyltransferase antibodies in the presence of 0.25% (W/V) SDS and 1.0% Triton X-100 as described (27) expect that protein A-Sepharose 4B (Pharmacia) was used as immunoadsorbent instead of formaldehyde-fixed Staphylococcus aureus cells. The immunoprecipitate was analyzed by SDS-polyaclyamide slab gel (10.0%) electrophoresis followed by fluorography (28).

RESULTS

Specificity of Anti-UDP-Glucuronyltransferase Immunoglobulin.

Effects of polyclonal antibodies against the purified transferase on various microsomal UDP-glucuronyltransferase activities and microsomal NADPH-cytochrome P-450 reductase are shown in Fig. 1. The activities toward 1-naphthol and 4-nitrophenol of the purified transferase were inhibited by the addition of anti-transferase immunoglobulin to the assay medium. Glucuronidation activities in microsomes toward 1-naphthol, 4-nitrophenol, and 5-hydroxytryptamine which were conjugated by the purified transferase were also inhibited by the anti-transferase immunoglobulin, but that toward 4-hydroxybiphenyl which could not be conjugated by the purified transferase could not be inhibited by the antibodies. Microsomal NADPH-cytochrome P-450 recuctase activity could neither be inhibited by the antibodies. Immunoblotting analysis showed that the antibodies selectively bound to a protein having a molecular weight of 54,000 (the transferase isozyme) among various microsomal proteins (Fig. 2). These results indicate that the antibodies was highly specific.

Induction of UDP-glucuronyltransferase.

Effects of administration of various inducers of drug metabolizing enzymes to rats on various activities of UDP-glucuronyltransferase in liver microsomes are shown in Table 1. 3-methylcholanthrene induced the glucuronidation activities toward 1-naphthol, 4-nitrophenol, and 5-hydroxytryptamine which are good substrates of the purified transferase isozyme.

Phenobarbital induced the glucuronidation activities toward 4-hydroxybiphenyl and bilirubin more effectively than those toward 1-naphthol, 4-nitrophenol, and 5-hydroxytryptamine, whereas PCBs induced the activities toward all substrates examined. This induction pattern is similar to those previously described (3). Glucuronidation activities toward some xenobiotics and also the endogenous substrate, 5-hydroxytryptamine, were increased in hyperplastic nodules which were prepared from a rat that had been dietarily administered with 0.05% 2-acetylaminofluorene as compared to those cells surrounding the nodule cells (Table I). However, bilirubin glucuronidation activity was suppressed by the 2-acetylaminofluorene-treatment (Table I). Fig. 3 shows the results of immunoblotting analysis of of liver microsomes from variously treated rats using anti-transferase immunoglobulin and antibodies against rat liver microsomal NADPH-cytochrome P-450 reductase which was not affected by 3-methylcholanthrene treatment. It was clear that the transferase protein was increased by 3-methylcholanthrene in the rat liver microsomes. The icrease in the transferase protein was found to be about 5-fold based on measurements of radioactivities associated with respective bands. This increase in the transferase protein is comparable with that in UDP-glucuronyltransferase activities toward 1-naphthol and 4-nitrophenol (about 5-4 fold) caused by 3-methylcholanthrene administration. Beside 3-methylcholanthrene, other inducers of drug metabolizing enzymes, such as phenobarbital and polychlorinated biphenyls, also caused increases in microsomal content of the transferase isozyme protein, though not so effectively as observed with 3-methylcholanthrene (Fig. 2).

It is known that dietary administration of 0.05% 2-acetylaminofluorene to rats for 6 weeks caused the formation of hyperplastic nodule cells in the liver. As shown in Table I, microsomes prepared from the nodule cells showed higher UDP-glucuronyltransferase activities toward 1-naphthol and 4-nitrophenol as compared with the activities of microsomes from healthy cells surrounding the nodule cells. Immunoblotting analysis showed that this increase in the activities in the nodule cell microsomes was also due to an increase in the content of the transferase isozyme (Fig. 4).

As shown in Table II, UDP-glucuronyltransferase activity using 4-nitrophenol as glucuronate acceptor was found to be present not only in liver microsomes but also in microsomes from small intestine, kidney and lung, though at lower levels than in hepatic microsomes. Interestingly, these extrahepatic activities were also induced by 3-methylcholanthrene administration (about 4-fold in kidney and about 3-fold in intestine and lung). Phenobarbital administration also caused slight induction of these extrahepatic UDP-glucuronyltransferase activities. Immunoblotting studies, shown in Fig. 5, indicated that the transferase isozyme protein could actually detected in kidney and lung microsomes, but not in brain and intestinal microsomes. The isozyme protein could, however, be detected in intestinal microsomes after administration of 3-methylcholanthrene or phenobarbital (Fig. 6). These treatments also increased the microsomal content of the isozyme in kidney and lung (Fig. 6).

DISCUSSION

The polyclonal antibodies raised against the UDP-glucuronyltransferase isozyme purified in "PART I" of this thesis recognized a single protein among a number of microsomal proteins of rat liver. Although Pfeil and Bock (11) have reported that their antibodies against the seemingly identical transferase isozyme precipitated the antigen but did not inhibit its activities, our antibodies did inhibit glucuronidation activities toward 1-naphthol, 4-nitrophenol, and 5-hydroxytryptamine of both liver microsomes and the purified enzyme. It is likely that the antibodies prepared by Pfeil and Bock (11) recognized antigenic sites that are distant from the active center of the enzyme. In accordance with the view that the glucuronidation of 4-hydroxybiphenyl is catalyzed by a transferase isozyme that is different from that purfied here and by Bock and coworkers (3), the activity of rat liver microsomes to catalyze the glucuronidation of 4-hydroxybiphenyl was not inhibited by the antibodies prepared in this study. This provided firm evidence that different isozymes are functional in glucuronidation of 1-naphthol, 4-nitrophenol, and 5-hydroxytryptamine on one hand and 4-hydroxybiphenyl on the other.

It has been generally believed that UDP-glucuronyltransferase activities toward endogeneous substrates such as testosterone, estrone, and bilirubin cannot be induced by administration of such inducers as 3-methylcholanthrene, phenobarbital and polychlorinated biphenyls. However, the results described above indicated that the activity of liver microsomes to conjugate 5-

hydroxytryptamine, an endogenous substrate, can be induced by these inducers, e.g. 2-fold by 3-methylcholanthrene, 3.4-fold by polychlorinated biphenyls, and 3.4-fold by 2-acetylaminofluorene. This is conceivable from the fact that the transferase isozyme purified in PART I" catalyzes the glucuronidation of 5-hydroxyotryptamine in addition to phenolic xenobiotics. In agreement with the finding reported by Bock et al. (10) and Pfeil and Bock (11), our results also indicate that the induction of rat liver UDP-glucuronyltransferase activity toward phenolic substrates such as 4-nitrophenol is accompanied by an immunochemically quantifiable increase in a single transferase isozyme (Table I and Figs. 2 and 3). Pfeil and Bock (7) have reported based on rocket immunoelectrophoresis analysis that their 3-methylcholanthrene-inducible isozyme of UDP-glucuronyltransferase cannot be induced by phenobarbital treatment. In the present study, however, this treatment could induce liver microsomal activities to conjugate 1-naphthol, 4-nitrophenol, and 5-hydroxytryptamine and this induction was accompanied by an increase in the isozyme protein identifiable by immunoblotting analysis (Fig. 2). Phenobarbital treatment has also shown to increase the amount of a UDP-glucuronyltransferase protein in chick embryonic liver (12). The reason for the failure of Pfeil and Bock (11) to detect any induction by phenobarbital treatment is unclear, but this might be related to changes in membrane characteristics caused by phenobarbital administration (5). The transferase isozyme in question was also increased by treatment with polychlorinated biphenyls (Fig. 4) and in hyperplastic nodule cells produced by 2-acetylaminofluorene treatment (Table I and

Fig. 5). Bock et al. (10) have suggested that the nodule cells are formed to offer resistance to cytotoxic agents such as carcinogens. In any case, the transferase isozyme seems to be markedly flexible and inducible by a variety of xenobiotics. This suggestes that the isozyme plays an important role in detoxication reactions.

Altough the mechanism of induction of UDP-glucuronyltransferase by administration of xenobiotics is not yet well understood, the data described in this paper indicate that the 3-methylcholanthrene induction of the activities toward 4-nitrophenol, 1-naphthol, and 5-hydroxytryptamine in rat liver microsomes results from an increase in the level of translatable mRNA coding for the transferase isozyme in question (Fig. 8). Increased levels of UDP-glucuronyltransferase mRNAs have also been reported for phenobarbital treatment of chick embryos (12) and 3-methylcholanthrene treatment of mice (29).

UDP-glucuronyltransferase activities have been detected in microsomes from several extrahepatic organs (5). Chowdhury et al. (30) have immunochemically detected the presence of UDP-glucuronyltransferase proteins in jejunum, kidney and adrenal gland cells using antibodies that recognize three isozymes of the transferase having molecular weights of 52,000, 53,000, and 56,000. In this study, UDP-glucuronlytransferase activities toward phenolic xenobiotics and the endogenous substrate, 5-hydroxytryptamine, were detected in microsomes from rat kidney, lung, and intestine and these activities were found to be induced by administration of 3-methylcholanthrene and some other xonobiotics (Table II and Figs. 6 and 7). The induction in these

extrahepatic organs was also shown to be accompanied by increase in the transferase isozyme protein (Fig. 7). Although no experiments have as yet been done, it is likely that the increase in the isozyme protein is a result of elevated levels of the isozyme mRNA in these organs.

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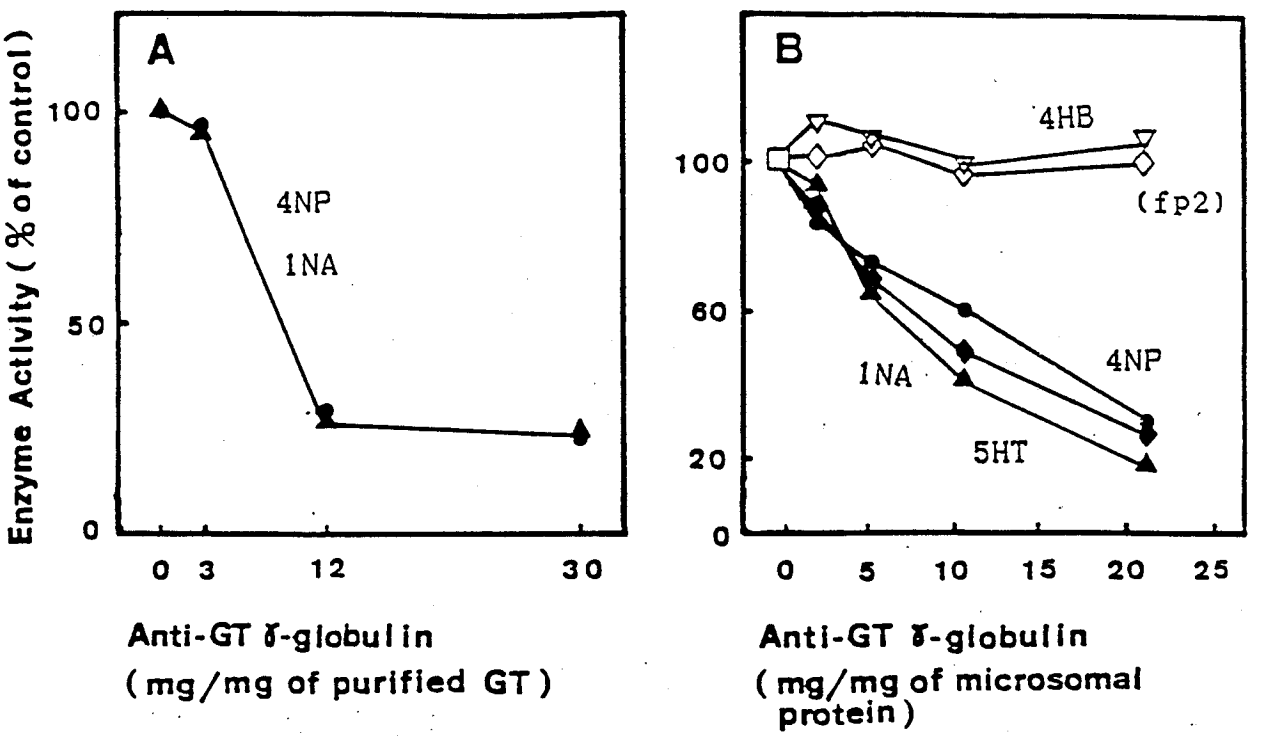

Fig. 1 Effects of anti-UDP-glucuronyltransferase immunoglobulin on UDP-glucuronyltransferase and NADPH-cytochrome P-450 reductase activities. Purified UDP-glucuronyltransferase (100 μg) (A) and solubilized microsomes (B) from 3-methylcholanthrene-treated rat liver were incubated overnight at 4°C in 20 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM DTT, 0.12% sodium cholate, 4% glycerol, 0.04% Emulgen 911 with various amounts of anti-UDP-glucuronyltransferase immunoglobulin . Enzyme activities were assayed as described in "MATERIALS AND METHODS". Specific activities of UDP-glucuronyltransferase toward 4-nitrophenol (●) and 1-naphthol (▲) are 4,716 and 4,682 nmol/min/mg of purified enzyme protein, respectively (A). Specific activities of UDP-glucuronyltransferase in microsomes (100%, toward 4-nitrophenol (●) , 1-naphthol (▲), 5-hydroxytryptamine (◆), and 4-hydroxybiphenyl (▽) are 51.1, 94.7, 27.5, and 26.2 nmol/min/mg of protein, respectively (B). NADPH-cytochrome P-450 reductase activity (◇) was 57.4 nmol/min/mg of protein (B).

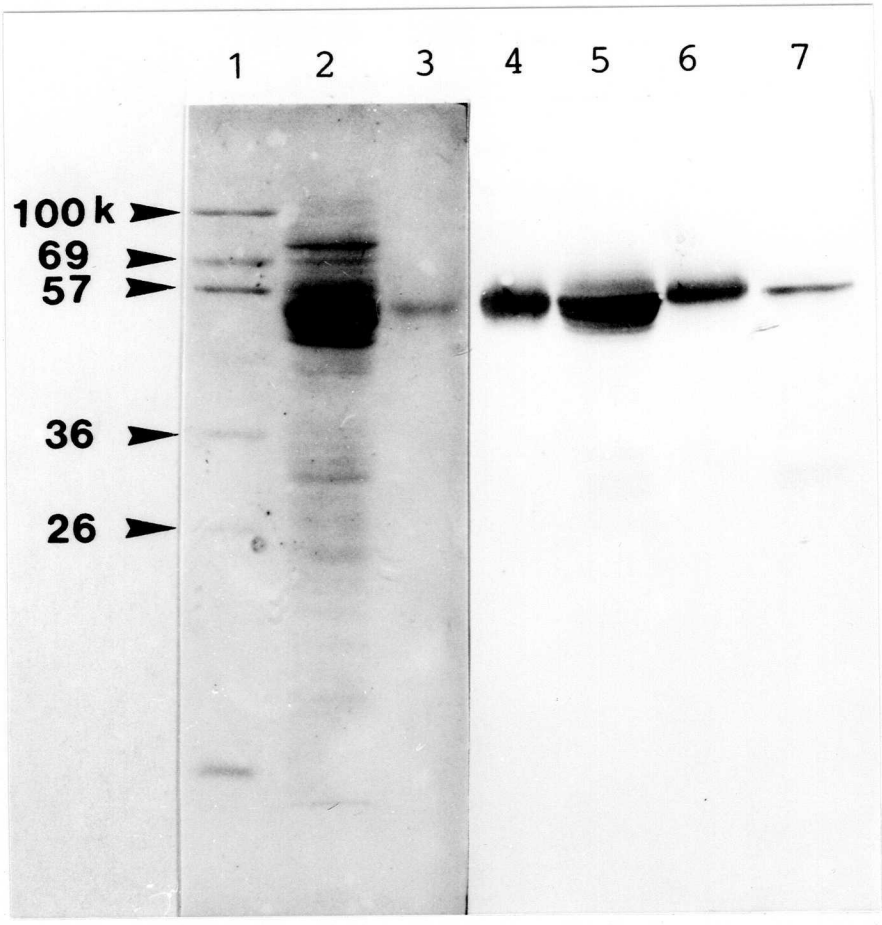

Fig. 2 SDS-Polyacrylamide gel electrophoresis and immunoblotting analysis of microsomal proteins and pure UDP-glucuronyltransferase with anti-UDP-glucuronyltransferase immunoglobulin. SDS-Polyacrylamide gel electrophoresis and electroblotting of proteins to a nitrocellulose sheet were performed as described in "MATERIALS AND METHODS". The proteins on the nitrocellulose sheet were stained with Amido Black (lane 1: marker proteins of molecular weight , lane 2: 10 µg of microsomal proteins from 3-methylcholanthrene-treated rat liver, lane 3: 1 µg of purified UDP-glucuronyltransferase) followed by enzyme-linked immunostaining with anti-rabbit IgG-peroxidase conjugates (1 µg of pure UDP-glucuronyltransferase (lane 4), 10 µg of microsomal proteins from 3-methylcholanthrene-treated (lane 5), phenobarbital-treated (lane 6), and untreated (lane 7) rat liver).

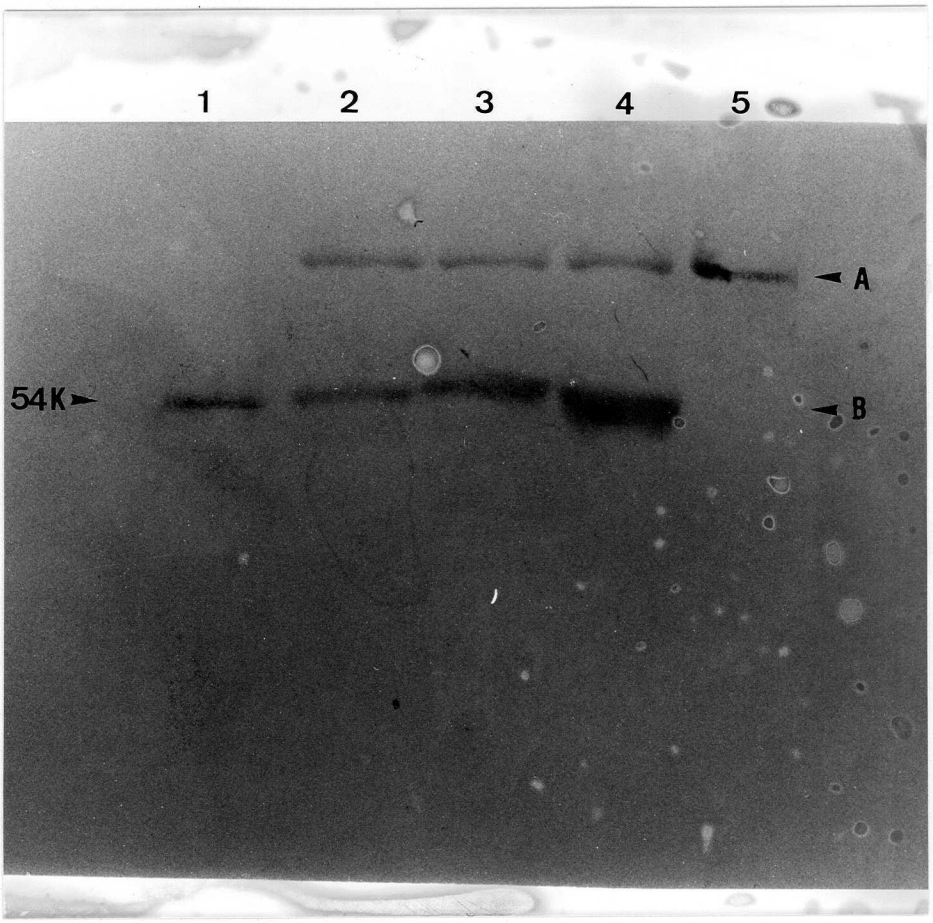

Fig. 3 Immunoblotting analysis of microsomal proteins with anti-UDP-glucuronyltransferase and anti-NADPH-cytochrome P-450 reductase immunoglobulins. Pure UDP-glucuronyltransferase (lane 1: 0.1 μg), pure NADPH-cytochrome P-450 reductase (lane 5: 0.1 μg) from rat liver, and hepatic microsomal proteins from untreated (lane 2: 5 μg), eugenol-treated (lane 3: 5 μg) and 3-methylcholanthrene-treated (lane 4: 5 μg) rats were subjected to SDS-polyacrylamide gel electrophoresis, immunoblotting with both of anti-UDP-glucuronyltransferase and anti-NADPH-cytochrome P-450 reductase immunoglobulins followed by binding with ^{14}C-labeled protein A (2.1 mCi/nmol), and fluorography for 3 days at -70°C. Eugenol (200 mg) was dissolved in 1 ml of olive oil and orally administered to rats 4 times for 2 days. Rat liver microsomes were prepared as described in "MATERIALS AND METHODS".

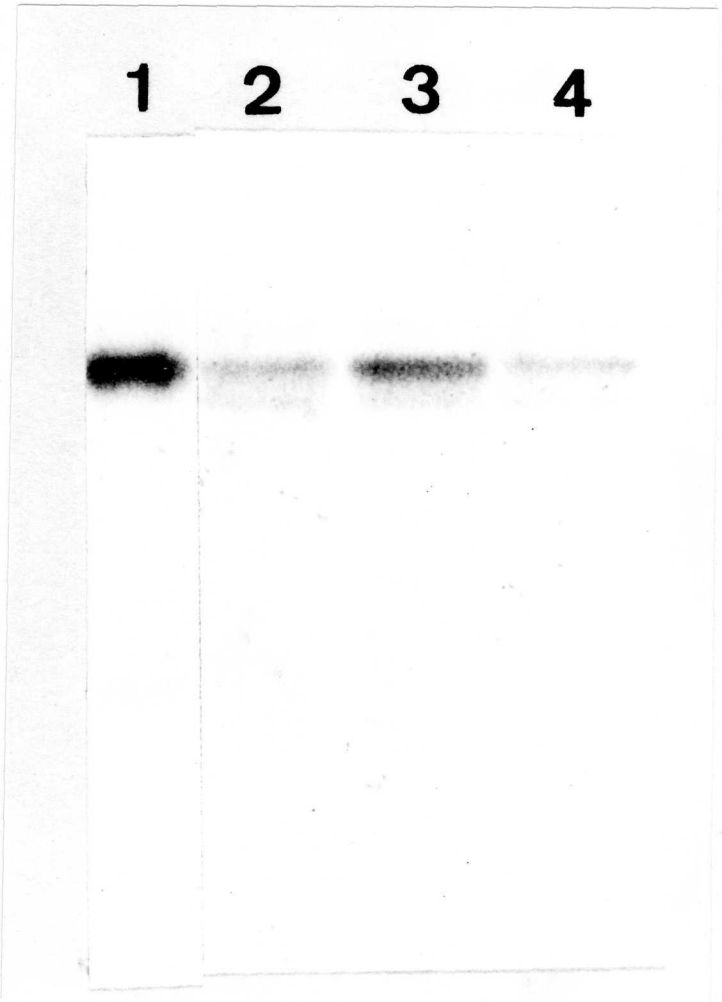

Fig. 4 Immunoblotting of microsomal protein from 2-acetylaminofluorene-treated rat livers with anti-UDP-glucuronyltransferase immunoglobulin. Lane 1: pure UDP-glucuronyltransferase (0.5 μg): lanes 2 and 4: microsomal protein (5 μg) from the cells around hyperplastic nodule cells, lane 3: microsomal protein (5 μg) from hyperplastic nodule cells.

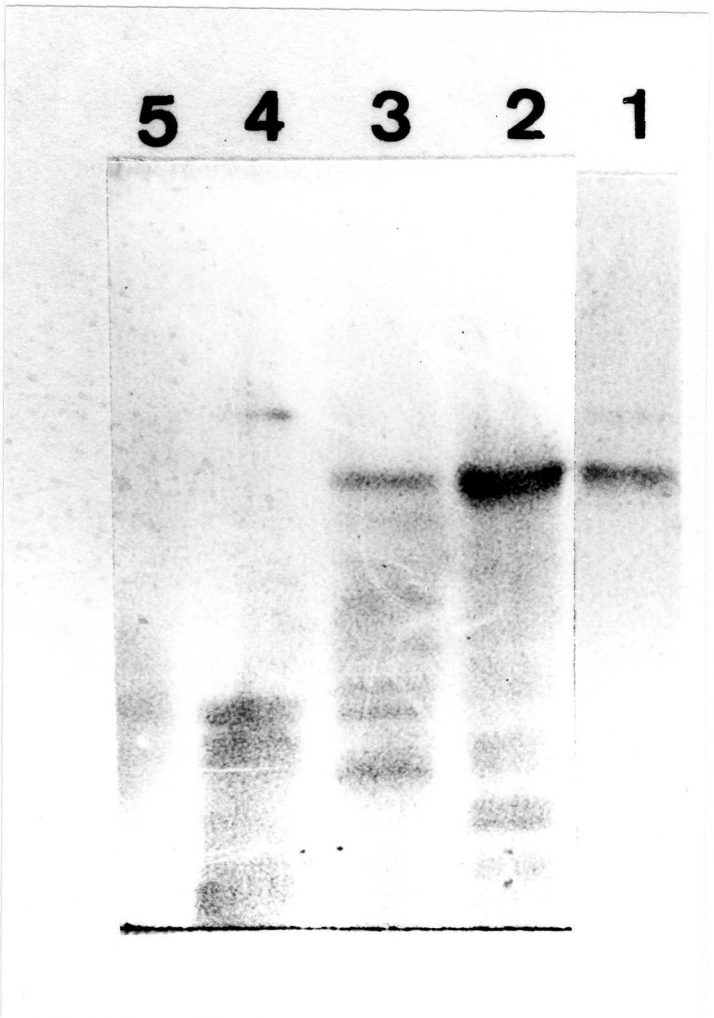

Fig. 5 Immunoblotting of microsomal proteins from rat liver, kidney, lung, intestine, and brain with anti-UDP-glucuronyltransferase immunoglobulin. The microsomal protein from liver (lane 1: 5 μg), kidney (lane 2: 10 μg), lung (lane 3: 10 μg), intestine (lane 4: 10 μg), and brain (lane 5: 10 μg).

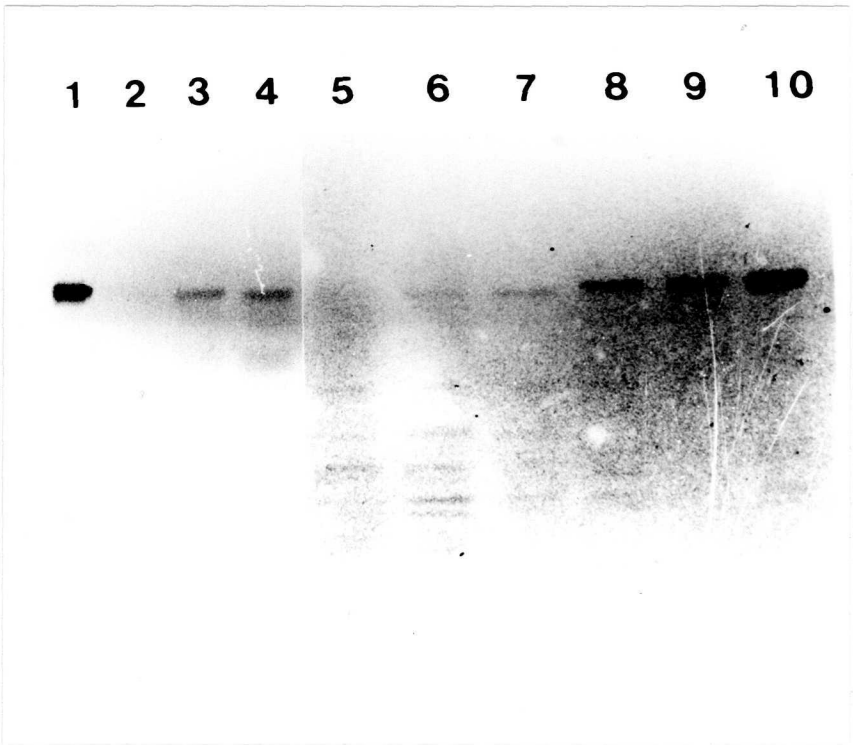

Fig. 6 Immunoblotting of kidney, lung, and intestine microsomal proteins from untreated, phenobarbital-treated, and 3-methylcholanthrene-treated rats. Lane 1: pure UDP-glucuronyltransferase (0.2 μg), lanes 2, 3, and 4: intestine microsomal protein (7.0 μg), lanes 5, 6, and 7: lung microsomal protein (7.0 μg), lanes 8, 9, and 10: kidney microsomal protein (7.0 μg). Microsomal proteins were from untreated (lanes 2, 5, and 8), phenobarbital-treated (lanes 3, 6, and 9), and 3-methylcholanthrene-treated (lanes 4, 7, and 10) rats.

Fig. 7 Analysis of the *in vitro* translation products of total RNA from rat liver by SDS-polyacrylamide gel electrophoresis. Total RNAs from untreated (lanes 1, 2, and 4) and 3-methylcholanthrene-treated (lane 3) rat livers were translated *in vitro*. The translation products were analyzed by SDS-polyacrlyamide gel electrophoresis and fluorography after precipitation with anti-UDP-glucuronyltransferase immunoglobulin in the absence (lane 1) and in the presence (lane 2) of 5 μg of unlabeled purified UDP-glucuronyltransferase or with unimminoglobulin (lane 4). Total RNA was omitted from the *in vitro* tsranslation system as a control (lane 5).

Table 1. Effects of some inducers on UDP-glucuronyltransferase activity in rat liver microsomes.
Enzyme activity was determined in liver microsomes, fully activated by the addition of sodium cholate (0.025 %).
Values represent the mean ± S.D. for 7 rats.

Inducers	UDP-glucuronyltransferase activity (nmol/min/mg of protein)									
	4-Nitrophenol		1-Naphthol		5-Hydroxytryptamine		4-Hydroxybiphenyl		Bilirubin	
None	26.8±1.3	(100)	16.5±1.0	(100)	14.2±1.5	(100)	26.5± 2.0	(100)	0.92±0.09	(100)
3-Methylcholanthrene	131.4±1.8	(490)	63.5±1.2	(385)	27.2±4.2	(195)	23.1± 1.7	(87)	1.41±0.10	(153)
Phenobarbital	60.8±3.7	(227)	36.0±0.7	(218)	20.7±2.0	(146)	127.0±13.8	(479)	1.80±0.14	(196)
PCBs	115.5±4.0	(431)	56.7±2.1	(344)	48.1±3.7	(339)	118.2±19.6	(446)	1.43±0.16	(155)
Nudule *	76.3	(285)	51.7	(313)	47.9	(337)	40.8	(154)	0.48	(52)
Around nodule *	70.6	(263)	32.0	(194)	39.7	(280)	47.4	(179)	0.71	(77)

* prepared from hyperplastic nodule cells (HN) or around of HN which were collected from livers of 7 animals treated with AAF.

( ) % of control.

Table II. Effects of some inducers on UDP-glucuronyltransferase activity toward 4-nitrophenol in the rat liver, kidney, lung and intestine microsomes. Enzyme activity was determined in liver, intestine, kidney and lung microsomes, fully activated by the addition of sodium cholate (0.025 %). Values represent the mean ± S.D. for 7 rats.

Inducers	UDP-glucuronyltransferase activity (nmol/mg of protein)			
	Liver	Intestine	Kidney	Lung
None	26.8 ± 1.3 (100)	9.78 ± 0.26 (100)	4.16 ± 0.24 (100)	3.33 ± 0.19 (100)
Phenobarbital	60.8 ± 3.7 (227)	13.48 ± 0.18 (138)	4.62 ± 0.50 (111)	5.35 ± 0.24 (161)
3-Methyl-cholanthrene	131.4 ± 1.8 (490)	26.00 ± 1.74 (265)	18.18 ± 1.61 (437)	9.01 ± 1.00 (270)

( ), % of control

PART III

TOPOLOGY OF THE UDP-GLUCURONYLTRANSFERASE IN MICROSOMAL MEMBRANE.

SUMMARY

The UDP-glucuronyltransferase isozyme purified in "PART I" bound to concanavalin A (Con A) on nitrocellulose sheets, and this binding was inhibited by α-methylmannoside. The transferase did not bind to Con A any more after treatment with endoglycosidase H (Endo H). Wheat germ agglutinin, on the other hand, was not capable of binding the transferase. These results provided evidence that the transferase is a glycoprotein carring "high mannose type" of oligosaccharide chain(s). UDP-glucuronyltransferase activities toward 1-naphthol, 1-nitrophenol, 5-hydroxytryptamine, 4-hydroxybiphenyl, and bilirubin in solubilized microsomes could be adsorbed to Con A-Sepharose 4B. Western blot analysis also indicated that the transferase protein in solubilized microsomes could bind to Con A-Sepharose 4B. However, these activities and the transferase protein in intact or sonicated microsomes could not be adsorbed to Con A-Sepharose 4B. It was concluded that the oligosaccharide chains of the transferase are exposed only to the luminal inner space of microsomal vesicles. Polyclonal antibodies raised against the transferse could bind to the outer surface of intact microsomal vesicles. When the complex consisting of intact microsomes, the antibodies and protein A-Sepharose 4B were well washed and then treated with 1.0% sodium cholate, the 1-naphthol conjugation activity could be restored. When intact microsomes were treated with trypsin, the transferase molecule in the membrane was cleaved into a 46,000-dalton fragment still bound to the microsomal membrane fraction and 8,000- and 7,000-dalton

polypeptides which were released to the supernatant fraction. These results indicated that the transferase is a transmembrane glycoprotein which spans the microsomal membrane extruding the "high mannose type" of oligosaccharide chain(s) to the luminal space of the vesicle.

INTRODUCTION

Glucuronidation catalyzed by UDP-glucuronyltransferase [EC 2,4,1,17] represents one of the major conjugation reactions involved in the metabolic conversion of a variety of xenobiotics as well as endogenous compounds such as bilirubin and steroid hormones to polar water-soluble products (3). Recent protein purification work has established the existence of several isoenzymes having different subunit molecular weights (50-56K daltons) (4-7). One important property of UDP-glucuronyltransferase is that it is inducible by administration of certain drugs and this induction has been shown to be accompained by an increase in mRNA coding for UDP-glucuronyltransferase (2,8,9). Another important feature of the transferase is its latency in liver microsomes: its full activity can be detected only after the disruption of microsomal membranes by various treatments (3,10). Two models have so far been proposed to explain this latency. In one model it is suggested that the transferase is extensively "constrained" by membrane lipids and, therefore, can be activated by agents or treatments that remove the constraint by affecting the lipid environment of the enzyme (11,12). The second model suggests that the observed changes in activity, brought about by various perturbations, are a result of compartmentation of the transferase in microsomal membrane which thereby limits the access of both UDP-glucuronyltransferase acid and acceptor to the enzyme (13,14). For a better understanding of the latency, it would be essential to investigate the interaction of the purified transferase with phospholipids (2,10,12,15,16) and to elucidate the

topological orientation of the enzyme in microsomal vesicles (31). This paper reports that the UDP-glucuronyltransferase isozyme purified in "PART I" is a glycoprotein and presents evidence that this enzyme spans the microsomal membrane extruding the oligosaccharide chain(s) to the lumen of endoplasmic reticulum.

MATERIALS and METHODS

Materials --- The chemicals used were obtained from the following sources: Ovalbumin (type VII), α-methylmannoside and trypsin from Sigma Chemical Co. (St. Louis, MO), concanavarine A (Con A) from Boehringer Mannheim GmbH (Mannheim, Germany), Con A-Sepharose 4B from Pharmacia, Uppsala, Endo-β-D-N-acetylglucosaminidase H (Endo H), wheat germ agglutinin-horse radish peroxidase conjugate from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan), horse radish peroxidase from Toyobo Co., Ltd. (Osaka, Japan), and anti-rabbit IgG-peroxidase conjugate from Jackson Immuno-Research (Pennsylvania, PA). [^{14}C]-Protein A (2.1 mCi/nmol) was a generous gift from Dr. M. Nakamura. Liver microsomes were prepared from Wistar rats (about 200 g) treated with 3-methylcholanthrene (80 mg/kg of body weight, intraperitoneally single dose) as described (1). A UDP-glucuronyltransferase showing high activities toward 4-nitrophenol was purified from liver microsomes of 3-methylcholanthrene-treated rats and antibodies to this enzyme was prepared as described (1,2).

Detection of Oligosaccharide Chain(s) --- Samples tested was subjected to SDS-polyacrylamide slab gel (10%) electrophoresis by the method of Laemmli (17). The protein bands separated on the gel were transferred to nitrocellulose sheets in 25 mM Tris solution containing 192 mM glycine and 20% methanol (v/v) essentially as described by Towbin et al. (18). The detection of oligosaccharide chains in the blotted protein bands was performed as previously described by Kijimoto-Ochiai et al. (19). The nitrocel-

lulose sheet carrying blotted protein bands was washed with 15 mM potassium phosphate buffer (pH 7.5) containing 0.05% Tween 80 ("Solution A") and soaked in a Con A solution (100 μg/ml in 15 mM potassium phosphate buffer, pH 7.5 for 1 h at room temperature. The sheet was then washed with Solution A for 1 h with several changes of the solution. The sheet thus washed was reacted with a horse radish peroxidase solution (100 μg/ml in 15 mM potassium phosphate buffer, pH 7.5) for 1 h at room temperature. This was washed in Solution A with several changes of the solution and then reacted with 10 ml of a 3,3'-diaminobenzidine solution (0.5 mg/ml in 15 mM potassium phosphate buffer, pH 7.0) containing 5 μl of 30% hydrogen peroxide at room temperature. After suitable color development (usually 2-5 min), the sheet was washed thoroughly with water. When wheat germ agglutinin-peroxidase was used for detection of oligosaccharide chain(s), the staining was conducted in the same way except that the peroxidase addition step was omitted.

Treatment with Endo-β-D-N-acetylglucosaminidase H (Endo H)--- To the purified transferase (2 μg) dissolved in 200 μl of 0.1 M citrate-phosphate buffer (pH 5.0) was added 5 units of Endo H and the mixture was incubated for 20 h at 37°C. SDS-polyacrylamide gel electrophoresis, electroblotting, and detection of oligosaccharide chains in the treated sample was performed as described above and below.

Removal of Oligosaccharide Chains from Glycoproteins on Nitrocellulose Sheets --- A nitrocellulose sheet carring blotted protein bands was immersed for 3 h at 37°C in 50 mM citrate buffer (pH 5.0) containing 10 m units/ml of Endo H.

Binding of UDP-Glucuronyltransferase to Con A-Sepharose 4B. --- Intact rat liver microsomes or those solubilized with 0.6% sodium cholate suspended in 0.1 M potassium phosphate buffer (pH 7.4) were incubated with various amounts of Con A-Sepharose 4B for 30 min at 4°C. After incubation, the mixture was centrifuged at 35 x g for 5 min at 4°C (Only Con A-Sepharose 4B beads sediment under these conditions.), and an aliquot of the supernatant was assayed for UDP-glucuronyltransferase activity toward 4-nitrophenol as a substrate. The amount of Con A-Sepharose 4B is determined in terms of the Con A content.

Protease Digestion of Microsomes --- Intact microsomes (1 mg) suspended in 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM DTT were digested with trypsin (4.0 mg) at 4°C. for 24 h. To the reaction mixture was then added 1 ml of ice-cold 20% trichloroacetic acid and the precipitated microsomal proteins were collected by centrifugation. The proteins were subjected to SDS-polyacrylamide slab gel electrophoresis and the protein separated were transferred to a nitrocellulose sheet. The immunoreactive polypeptide bands on the sheet were detected by antibodies to the transferase isozyme with the aid of second antibodies conjugating horse radish peroxidase.

Analytical Procedure --- UDP-glucuronyltransferase activities toward various substrates were assayed by the methods described in respective references at indicated aglycone concentration : 0.5 mM 1-naphthol (20), 0.5 mM 4-nitrophenol (10), 0.5 mM 4-hydroxybiphenyl (21), 1.0 mM 5-hydroxytryptamine (22), 0.1 mM bilirubin (23). NADPH-cytochrome P-450 reductase was determined

by the method of Omura et al. (24). γ-Glutamyl transpeptidase activity was determined as previously described by Taniguchi et al. (25). Glucose-6-phosphatase was determined by the method of Swanson (26). Protein was determined by the method of Lowry et al. (27) using bovine serum albumin as a standard.

RESULTS

Nature of UDP-Glucuronyltransferase as Glycoprotein

The UDP-glucuronyltransferase isozyme purified in "PART I" and rat liver microsomes solubilized with 0.6% sodium cholate were subjected to SDS-polyacrylamide slab gel electrophoresis followed by electroblotting onto nitrocellulose sheets. Blotted protein bands were visualized by Amido Black staining. As shown in Fig. 1, solubilized microsomes displayed a number of polypeptides bands (lane 2), whereas the purified transferase isozyme exhibited only a simple band having a molecular weight of 54,000 (lane 3). When the blotted proteins were reacted with Con A and Con A binding to polypeptides was detected by peroxidase staining, it was found that many microsomal proteins bound to Con A (Fig. 1, lane 5) and the purified transferase also strongly bound to Con A (Fig. 1, lane 4). This suggested the occurence of mannose-containing oligosaccharide chain(s) in the transferase molecule. This was further supported by the fact that Con A bound to the transferase was inhibited by the addition of α-metylmannoside (compare lanes 2 and 4 of Fig. 2). To further examine the presence of mannose-containing oligosaccharide chain(s), the purified transferase was digested with Endo H both in solution and on nitrocellulose sheets. As shown in Fig. 2 (lanes 3 and 5), this treatment abolished the Con A-binding capacity of the transferase in both cases. When wheat germ agglutinin-peroxidase, instead of Con A-peroxidase, was used (19), the transferase failed to bind to this agglutinin (Fig. 3, lane 4), whereas ovalbumin, a glycoprotein containing "hybrid

type" of oligosaccharide chain(s), could bind to wheat germ agglutinin (Fig. 3, lane 3). From these results, it was concluded that the transferase is a glycoprotein containing "high mannose type" of oligosaccharide chain(s).

Oligosaccharise-bearing Site is Exposed to the Lumen of the Endoplasmic Reticurumm.

Intact liver microsomes and those solubilized with 0.6% sodium cholate were incubated with increasing amounts of Con A-Sepharose 4B. After precipitating the Con A-Sepharose 4B beads by brief centrifugation (35 x g, 5 min), UDP-glucuronyltransferase and some other microsomal enzymes activities remaining in the supernatant was measured (Fig. 4). Such microsomal enzymes as glucose-6-phosphatase and NADPH-cytochrome P-450 reductase could not be adsorbed from solution to Con A-Sepharose 4B in both intact and solubilized microsomes and thus remained in the supernatant (Fig. 4, A and C). The γ-glutamyl transpeptidase (γ-GTP), which is a glycoprotein located on the outer surface of plasma membrane and was present in the microsomal preparation as a contaminant, was adsorbed to Con A-Sepharose 4B even when the microsomes were not treated with sodium cholate (Fig. 4 A) as previously described (28). The finding that Con A-Sepharose 4B failed to bind to intact microsomes indicated that all mannose-containing oligosaccharide chain(s) of microsomal glycoproteins, including the transferase, are exposed to the luminal space of microsomal vesicles. In accordance to this notion UDP-glucuronyltransferase activities of intact microsomes toward 1-naphthol, 4-nitrophenol, 5-hydroxytryptamine, 4-hydroxybiphenyl, and bilirubin remained in the supernatant after the Con A-Sepharose 4B treatment. These ac-

tivities were, however, bound to Con A-Sepharose 4B when microsomes solubilized with sodium cholate were examined (Fig. 4 D). It was, therfore, likely that the "high mannose type" of oligosaccharide chain(s) of the transferase isozyme purified in "PART I" as well as the isozymes catalyzing 4-hydroxybiphenyl and bilirubin conjugation reactions are exposed to the luminal (inner) space of microsomal vesicles. The results of immunoblotting analysis of microsomal proteins treated with Con A-Sepharose 4B as described in Fig. 4 are shown in Fig. 5. The anti-transferase antibodies used has been reported to specifically recognize the transferase isozyme among various microsomal proteins on nitrocelulose sheets (2). The transferase molecule in sodium cholate-solubilized microsomes was adsorbed to Con A-Sepharose 4B and thus precipitated with Con A-Sepharose 4B beads (lanes 3 and 4), whereas the transferase in intact microsomes could not bind to Con A-Sepharose 4B and thus remained in the supernatant (lanes 1 and 2). The results shown in Figs. 4 and 5 indicated that the site in the transferase molecule carrying the oligosaccharide chain(s) is exposed to the luminal (inner) space of microsomal vesicles. As shown in Fig. 6, the transferase activity of intact microsomes was significantly activated by sonication in agreement with a previous report (10). However, the transferase activity in sonicated microsomes could not be adsorbed to Con A-Sepharose 4B and thus remained in the supernatant after precipitation of Con A-Sepharose 4B beads. As shown in Fig. 7, the tranferase activity of intact microsomes was fully activated by treatment with a concentration of sodium cholate as low as 0.1% as previously reported (10). However, the trans-

ferase activity of 0.1% sodium cholate-treated microsomes could not adsorbed to Con A-Sepharose 4B as in the case for sonication-activated microsomes. Practically no transferase activity was solubilized from microsomes in the presence of 0.1% sodium cholate. A small amout of the transferase was solubilized in the presence of 0.2% sodium cholate and completely solubilized with 0.5% sodium cholate as previously described (10). The ratio of transferase activity adsorbed to Con A-Sepharose 4B to the total activity in microsomes was corelated to the extent of solubilization of the activity from microsomes (Fig. 7). These results indicated that sonication and treatment with low concentration of sodium cholate (0.1%) abolish the latency of the transferase but can not disrupt the microsomal membrane completely.

Transmembrane Topology of UDP-Glucuronyltransferase

To examine whether or not a part of the transferase molecule is also extruted to the outer surface of microsomal vesicles, the following experiments were carried out. Intact microsomes were mixted with anti-transferase antibodies and incubated for 12 h at room temperature. The mixtures were then centrifuged at 105,000 x g for 1 h and the pellets were washed twice with 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM DTT ("Buffer A"). The washed pellets were resuspended in Buffer A and incubated with [^{14}C]-protein A for 4 h at room temperature. After centrifugation at 105,000 x g for 1 h, the pellets were washed with Buffer A suspended in Buffer A, dot-blotted to nitrocellolose sheets, and subjected to fluorography. As shown in Fig. 8 B, the amount of immunoglobulin bound to intact microsomes was increased as the amount of antibodies added was increased (Fig.

8). When the globulin fraction from an unimmunized rabbit was used, no binding of [^{14}C]-protein A was detected (Fig. 8 A). Counting of radioactivities in these spots confirmed this conclusion in a quantitative fashion (Fig. 8 C). Similar experiments were then carried out using protein A-Sepharose 4B instead of [^{14}C]-proein A. In this case, the pellet of intact microsomes to which antibodies were bound was washed twice with Buffer A as above, and incubated with protein A-Sepharose 4B at room temperature for 4 h. After centrifugation of the mixture consisiting of intact microsomes, antibodies, and protein A-Sepharose 4B at 35 x g for 5 min, the pellet was washed twice with Buffer A containing 1.0% sodium cholate and resuspended in Buffer A. As shown in Fig. 9, this suspension exhibited a UDP-glucuronyltransferase activity toward 1-naphthol and the activity was linearly dependent on the amount of the antibodies added to microsomes. When the globulin fraction prepared from an unimmunized rabbit was added, instead of the anti-transferase antibodies, microsomes were not precipitated. It was thus clear that the antibodies recognized a portion of the transferase molecule which is exposed to the outer surface of intact microsomal membrane.

In an attemp to gain information concerning the exposed portion of the transferase, intact microsomes were digested with trypsin at 4^{o}C for 1 or 24 h and the microsomes were subjected to SDS-polyacrylamide slab gel electrophoresis. The polypeptide bands thus separated were then analyzed by immunoblotting using the anti-transferase antibodies as a probe. As shown in Fig. 10, undigested microsomes gave a single immunoreactive band of the transferase having a molecular weight of 54,000. Upon trypsin

digestion, another immunoreactive band having a molecular weight of 46,000 appeared together with two faint bands of 7,000 and 8,000 daltons (Fig. 10, lanes 2 and 3). After digestion for 24 h, the suspension was centrifuged at 105,000 x g for 60 min and the pellet and supernatant were analyzed by immunoblotting. As can be clearly seen in Fig. 10 (lanes 5 and 6), the intact transferase and its 46,000-dalton fragment were still bound to microsomes, whereas the 7,000- and 8,000-dalton fragments were recovered in the supernatant. It could be concluded from these results that trypsin had cleaved a 8,000-dalton portion of the transferase and this portion must have been exposed to the outer surface of the microsomal membrane. The 7,000-dalton fragment seemed to have been produced from the 8,000-dalton fragment by further proteolysis.

DISCUSSION

Yuasa (10) has previously reported that UDP-glucuronyltransferase partially purified from rabbit liver microsomes is a glycoprotein based on the observation that upon SDS-polyacrylamide gel electrophoresis of the preparation the band corresponding to the transferase was positively stained with PAS reagent. In 1984 Mackenzie *et al.* (29) reported the nature of UDP-glucuronyltransferase as glycoprotein by an experiment in which the antigen immunoadsorbed from tritiated liver microsomes was digested with Endo H. More recently, Jackson *et al.* and Mackenzie (31) have determined the nucleotide sequences of cDNA clones encoding for hepatic UDP-glucuronyltransferase and shown that potential asparagine-linked glycosylation sites are contained in their primary structures. The results reported in this thesis provide direct evidence that the transferase isozyme purified in "PART I" contains asparagine-linked "high mannose type" of oligosaccharide chain(s).

Several transmembrane proteins of the endoplasmic reticulum such as ribophorins (32) and 3-hydroxy-3-metylglutaryl CoA reductase (33) have been shown to possess oligosaccharide chains that are extruded to the luminal space based on evidence obtained by Con A binding studies. Other microsomal proteins such as cytochrome b_5 (34) and NADPH-cytochrome P-450 reductase (35), which do not span the membrane, lack oligosaccharide chains. In the present study ample evidence was obtained to show that the "high mannose type" of oligosaccharide chain(s) attacted to the UDP-glucuronyltransferase isozyme are also extruded to the

luminal space of the endoplasmic reticulum. The finding that the transferase activities toward 4-hydroxybiphenyl and bilirubin, which are conjugated by isozymes other than that purified in this study, could also be adsorbed by Con A-Sepharose 4B suggests that these isozymes also contain "high mannose type" of oligosaccharide chain(s) on their portion exposed to the luminal space.

Since polyclonal antibodies raised against the UDP-glucuronyltransferase isozyme bind to intact microsomes, it is clear that a certain portion of the transferase molecule is exposed to the outer surface of the microsomal membrane. This portion seems to have a molecular weight of at least 8,000 because digestion of intact microsomes with trypsin releases 8,000- and 7,000-dalton fragments, both of which can be recognized by the polyclonal antibodies. Trypsin digestion also produces a 46,000-dalton fragment that is still tightly attached to microsomes. These observations indicated that this isozyme of UDP-glucuronyltransferase (M_r = 54,000) is a transmembrane protein that is exposed to both outer and inner spaces of the microsomal vesicle. The topology of this transferase isozyme in the microsomal membrane may be depicted as shown in Fig. 11.

It is well known that UDP-glucuronyltransferase shows latency in intact microsomes. In other words, its full activity can be detected only after perturbation of the microsomal membrane by such treatments as sonication and addition of suitable detergents (3, 10). Even though sonication results in full activation of the transferase activity in intact microsomes, Con A-Sepharose 4B is still inaccessible to the oligosaccharide chain(s) of the enzyme (Fig. 6), suggesting that disruption of microsomes by

sonication is sufficient for removing the latency of the transferase but not so extensive to allow the entry of Con A-Sepharose 4B inside the vesicle. Vessey and Zakim (11) have actually shown that sonication of microsomes allows UDP-glucuronyltransferase activity to become trypsin sensitive but the vesicle still remains impermeable to inulin. One possibility is that sonication causes a rearrangement of the environment of transferase leading to both activation of the enzyme and its susceptibility to protease but does not affect significantly the permeability of the membrane.

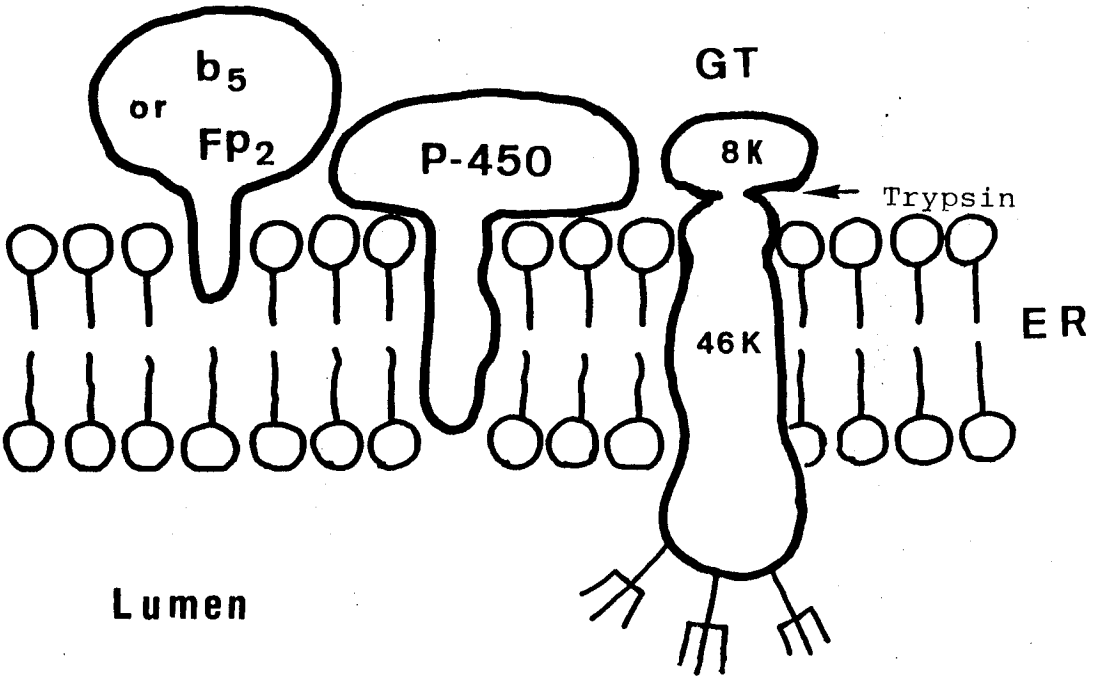
b5
or
Fp2
P-450
GT
8K
Trypsin
46K
ER
Lumen
"hight mannose type"
Oligosaccharide Chains

Fig. 11

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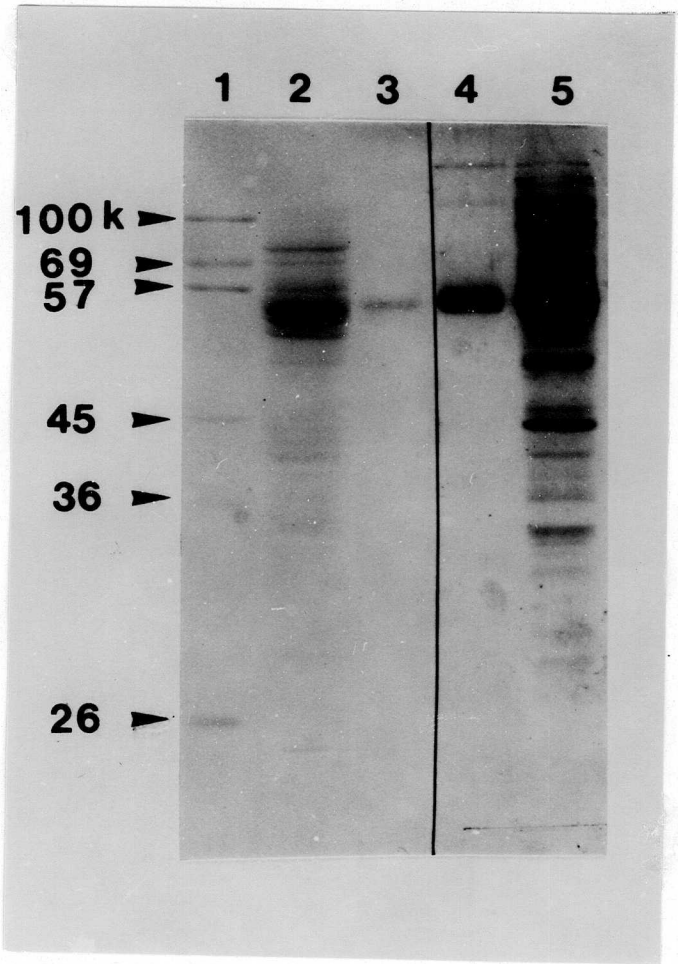

Fig. 1 Detection of oligosaccharide chain(s) with Con A and peroxidase. Microsomal proteins (lanes 2 and 5) and pure UDP-glucuronyltransferase (lanes 3 and 4) on SDS-polyacrylamide gels were electrophoretically transferred to nitrocellulose sheets. The nitrocellulose sheets were stained with Amido Black (lanes 1, 2, and 3) and treated with Con A and peroxidase (lanes 4 and 5) as described in "MATERIALS AND METHODS". Lane 1 indicates marker proteins of molecular weight (phosphorylase b : 97,400, bovine serum albumin: 67,000, pyruvate kinase: 57,400, ovalbumin: 45,000, lactate dehydrogenase: 36,000, chymotrypsinogen A: 25,700).

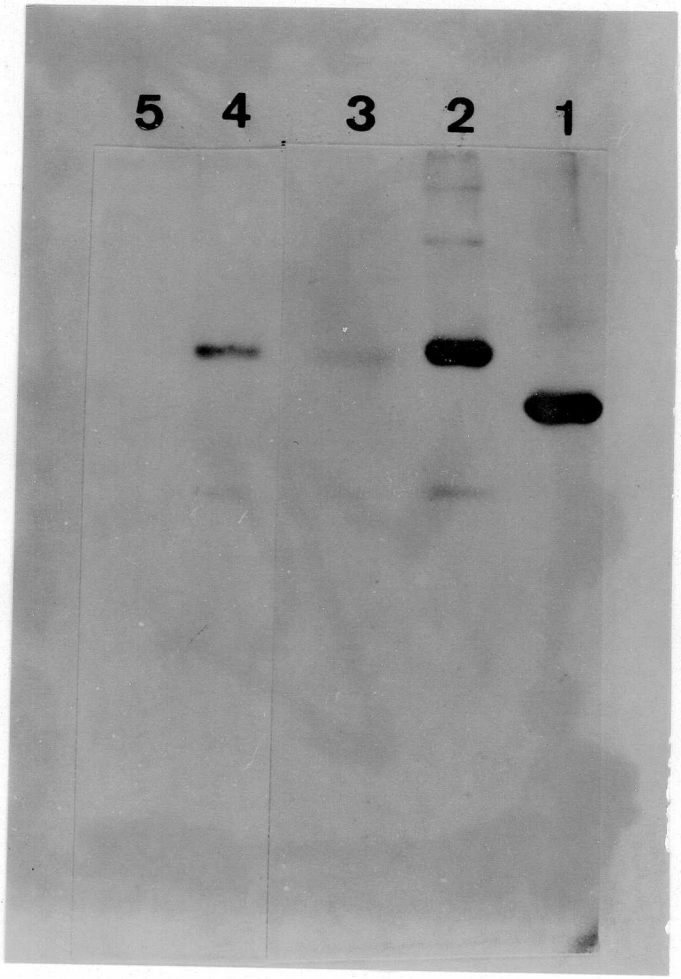

Fig. 2 Analysis of the structure of oligosaccharide chain(s) of UDP-glucuronyltransferase with endoglycosidase H and Con A. Ovalbumin (lane 1: 1 μg), pure UDP-glucuronyltransferase (lane 2: 0.5 μg), and endoglycosidase H-digested UDP-glucuronlytransferase lane 3: 0.5 μg) on the nitrocellulose sheet were treated with Con A as described in "MATERIALS AND METHODS". In case of lane 4, pure UDP-glucuronyltransferase (0.5 μg) on the nitrocellulose sheet was treated with Con A in the presence of 5 mM α-methylmannoside. In case of lane 5, pure UDP-glucronyltransferase on the nitrocellulose filter was digested with endoglycosidase H and then treated with Con A as described in "MATERIALS AND METHODS".

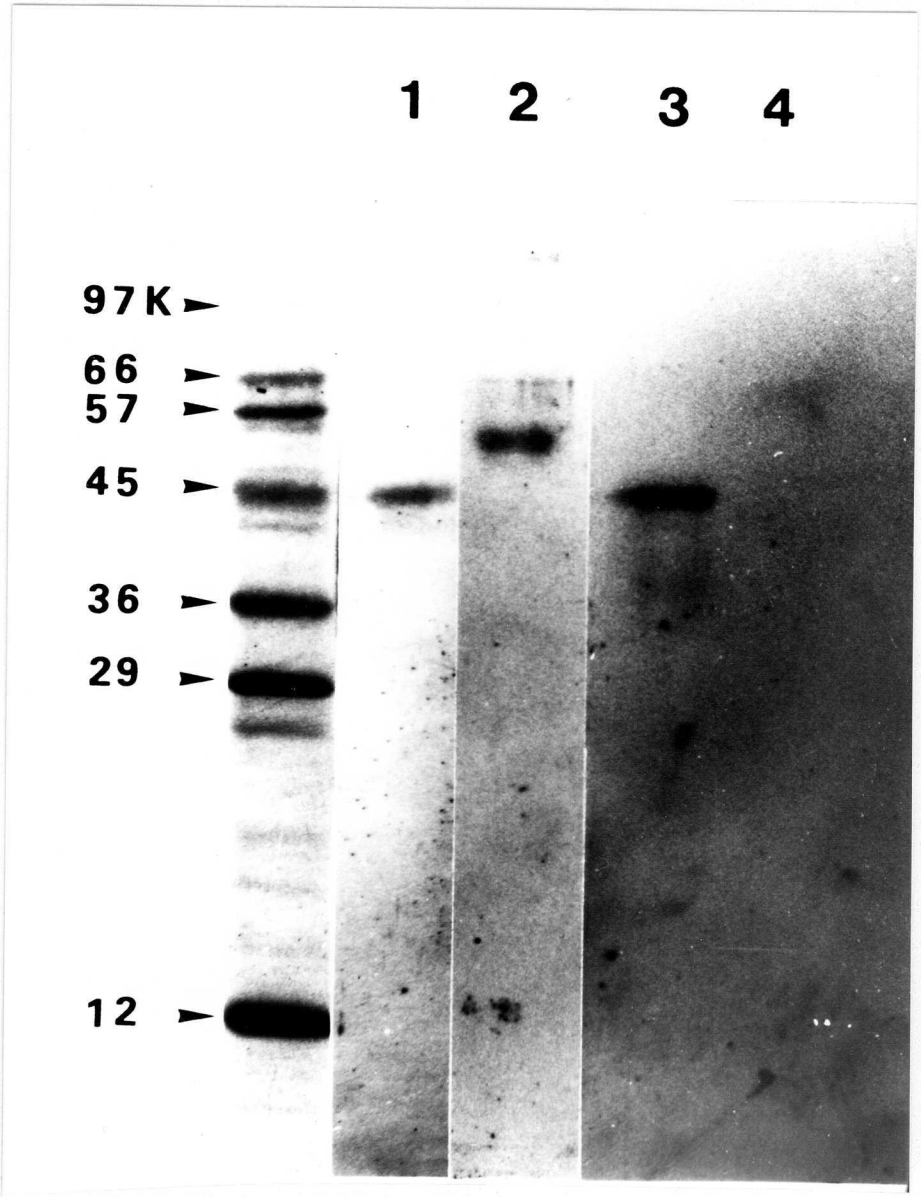

Fig. 3 Analysis of the structure of oligosaccharide chain(s) of UDP-glucuronyltransferase with wheat germ agglutinin-peroxidase. Ovalbumin (lane 1: 5 μg) and marker proteins of molecular weight (lane 2) on the nitrocellulose sheet were stained with Amido Black. Ovalbumin (lane 3: 5 μg) and pure UDP-glucuronyltransferase (lane 4: 5 μg) on the nitrocellulose sheet were treated with wheat germ agglutinin-peroxidase as described in "MATERIALS AND METHODS".

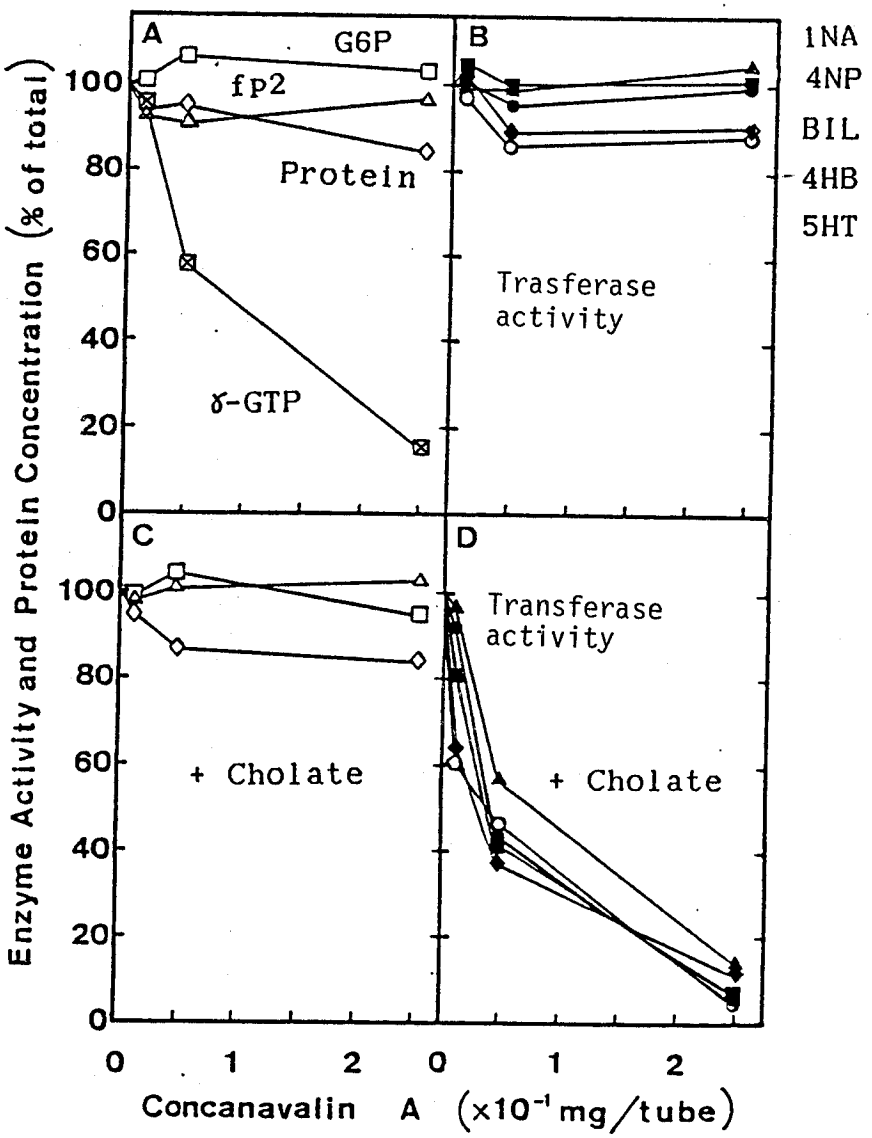

Fig. 4 Binding of UDP-glucuronyltransferase to Con A-Sepharose 4B. Hepatic microsomes from 3-methylcholanthrene-treated rats were incubated with the indicated amount of Con A-Sepharse 4B for 5 min at room temperature in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM DDT (A and B) or in the same buffer containing 0.6% sodium cholate (C and D). After incubation, all mixture were separately centrifuged at 35 x g for 2 min at 4°C, and aliquots of the supernatants were assayed for protein concentration and UDP-glucuronyltransferase activity. The amount of Con A-Sepharose 4B is expressed in terms of the Con A content. 1NA:1-naphthol, 4NP:4-nitrophenol, Bil:bilirubin, 4HB:4-hydroxybiphenyl, 5HT:5-hydroxytryptamine, G6P:glucose-6-phosphatase, fp2:NADPH-cytochrome P-450 reductase, -GTP: -glutamyl transpeptidase.

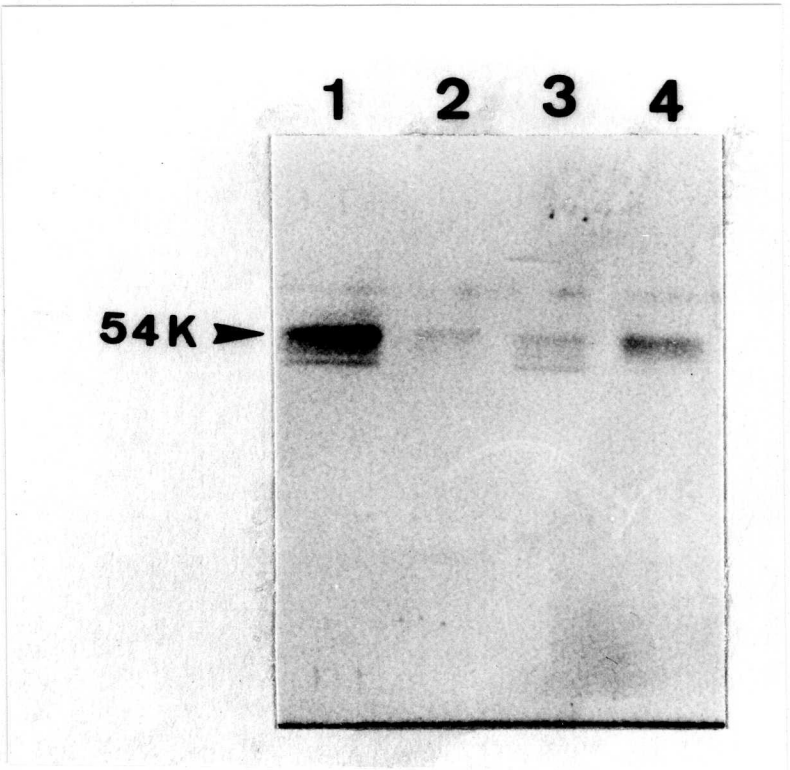

Fig. 5 Immunoblotting analysis of supernatants and precipitates after centrifugation of the mixture of microsomes and Con A-Sepharose 4B. Intact microsomes (0.8 mg of protein) (lanes 1 and 2) and microsomes (0.8 mg of protein) (lanes 3 and 4) disrupted with 0.6% sodium cholate from 3-methylcholanthrene-treated rat liver were mixed with Con A-Sepharose 4B (2.0×10^{-3} mg of Con A) and centrifugated as described in Fig. 4. Resultant supernatants (lanes 1 and 3) and precipitates (lanes 2 and 4) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting analysis as described in "MATERIALS AND METHODS".

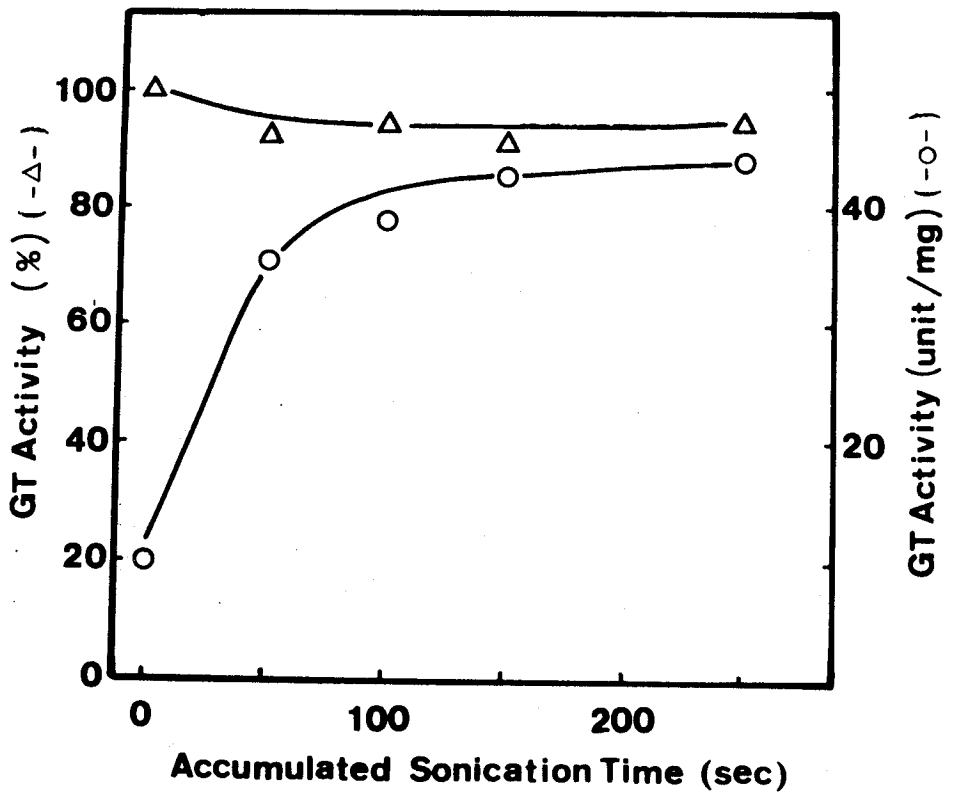

Fig. 6 Effects of sonication of microsomes on the binding of UDP-glucuronyltransferase to Con A-Sepharose 4B. Intact microsomes were suspended to a protein concentration of about 3.0 mg per ml in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM DTT and sonicated in a Branson Sonifire equipped with a standard 1/2" horn at a power level of 5.5. The sonication was conducted for 10 sec, followed by 50 sec of interval, and this procedure had been repeated until the indicated accumulated sonication time was reached. The activity of UDP-glucuronyltransferase in the sonicated microsomes was assayed toward 4-nitrophenol and shown as unit per mg of microsomal protein (O). The sonicated microsomes (0.8 mg of protein) were mixed with Con A-Sepharos 4B (0.2 mg of Con A) and then the mixture was centrifuged as described in Fig. 4. The UDP-glucuronyltransferase activity toward 4-nitrophonol in the resultant supernatant (Δ) was assayed and the data are indicated as percents relative to the whole activities in the sonicated microsomes before the addition of Con A-Sepharose 4B.

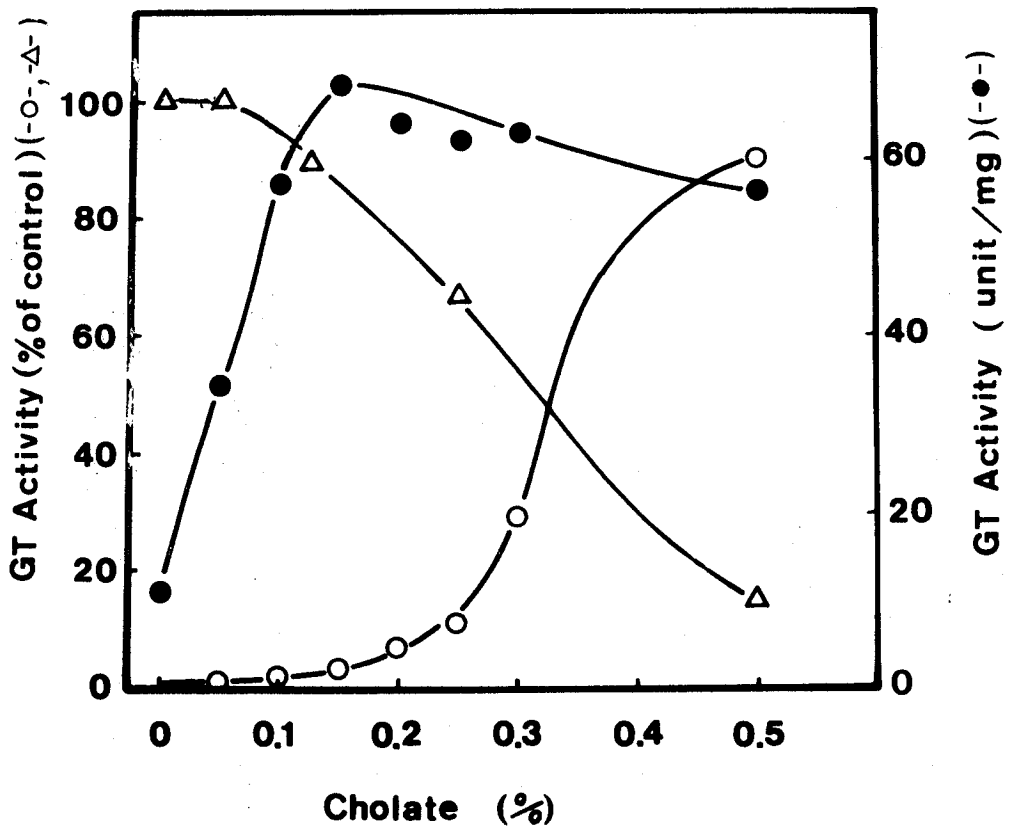

Fig. 7 Effects of the treatment of microsomes with sodium cholate on the binding of UDP-glucuronyltransferase to Con A-Sepharose 4B. Microsomes (3.0 mg of protein per ml) were treated with various concentrations of sodium cholate. Then, UDP-glucuronyltransferase activity in the cholate-treated microsomes was assayed (●). After centrifugation of the cholate-treated microsomes at 105,000 x g, the UDP-glucuronlytransferase activity in the supernatant (○) was assayed. The cholate-treated microsomes (0.8 mg of protein) were mixed with Con A-Sepharose 4B (0.2 mg of Con A) and centrifuged as described in Fig. 4. The UDP-glucuronyltransferase activity in the resultant supernatant (△) was assayed at 0.05% sodium cholate in the assay medium.

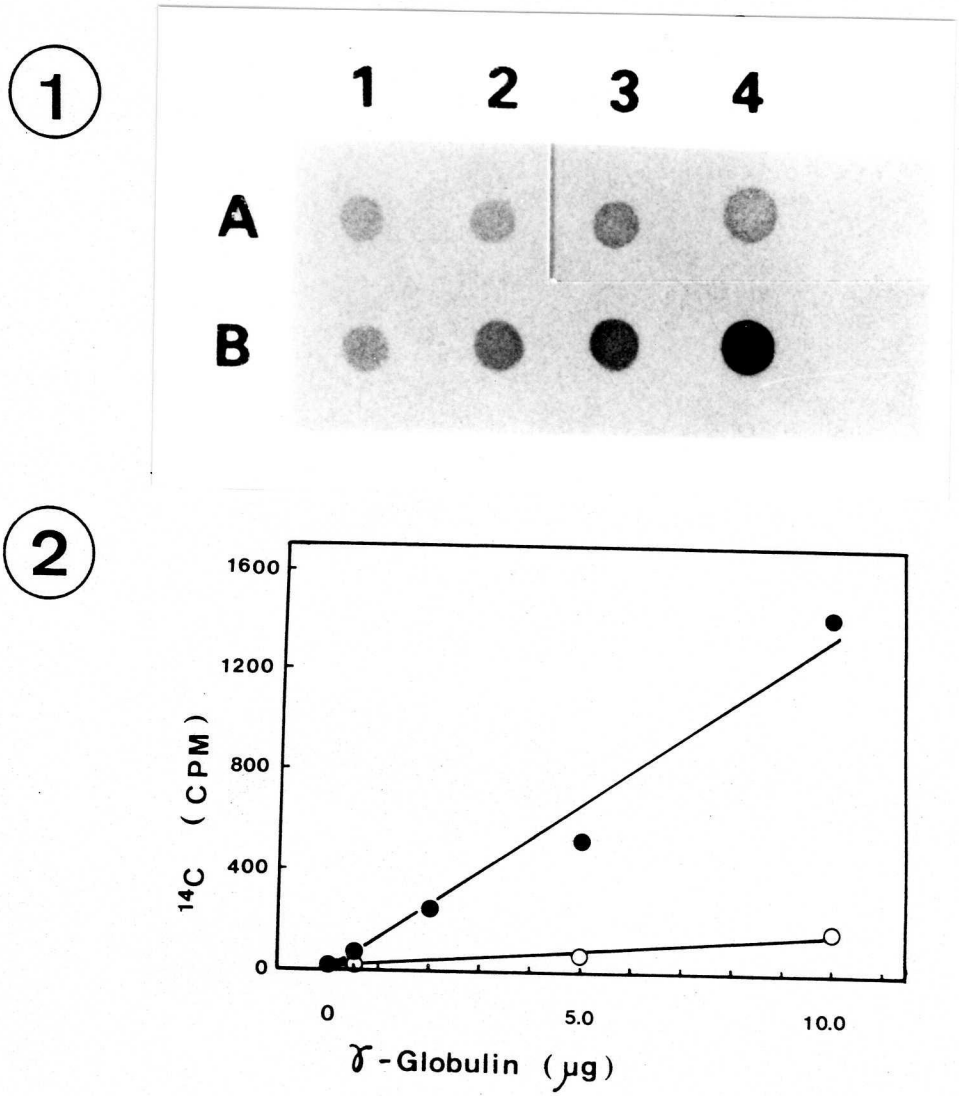

Fig. 8 Binding of anti-UDP-glucuronyltransferase immunoglobulin to microsomes. Intact microsomes (0.5 mg of protein) was incubated with indicated amounts of anti-UDP-glucuronyltransferase immunoglobulin for 12 h at room temperature and the mixtures were centrifuged at 105,000 x g for 60 min. The pellets were suspended in 0.2 ml of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM DTT (Buffer A) and washed twice with 0.2 ml of Buffer A. The pellets were separately suspended in 1 ml of Buffer A and incubated with 2 μl of ^{14}C-protein A (2.1 mCi/nmol) for 4 h at room temperature. After centrifugation of the mixture at 105,000 x g for 60 min. the pellets were washed with 1 ml of Buffer A and suspended in 0.2 ml of Buffer A. The suspensions (5 ml) were dott-blotted to a nitrocellulose sheet and fluorography (①). Radioactivities of these spots were counted (②). Anti-UDP-glucuronyltransferase immunoglobulin (1: B, 2: -●-) and unimmuno blobulin fraction (1: A, 2: -○-) were used.

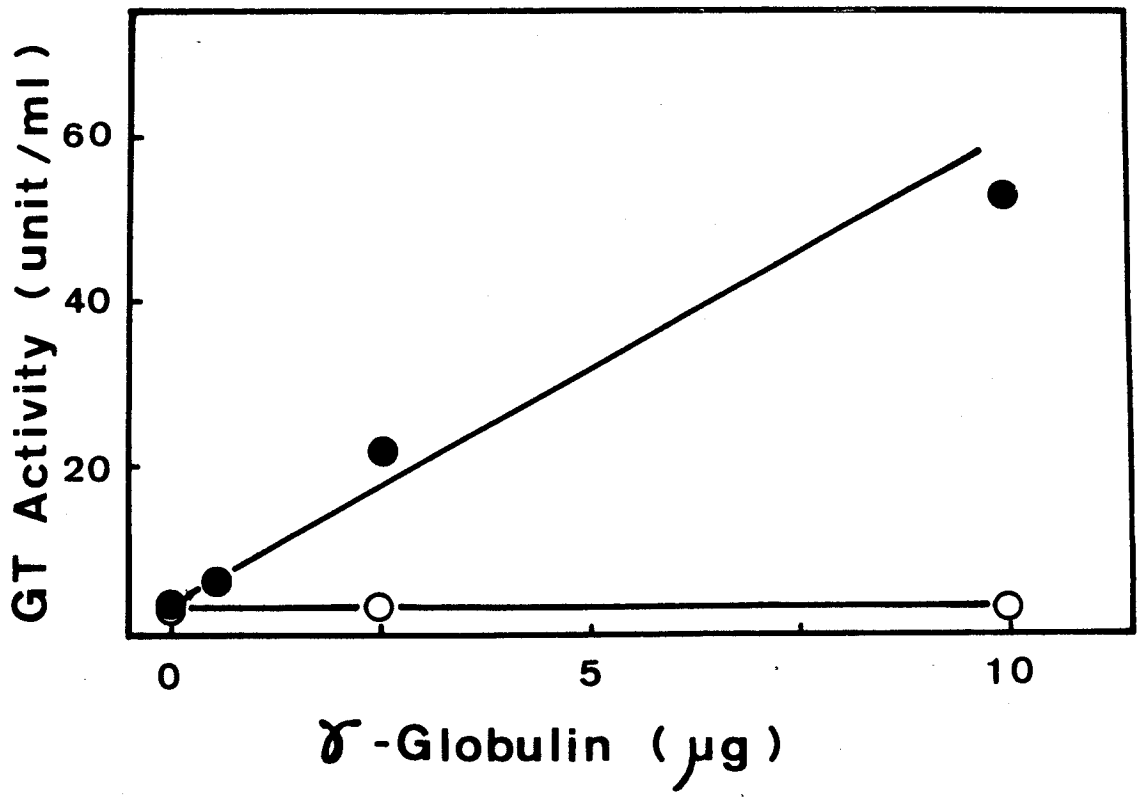

Fig. 9 Binding of anti-UDP-glucuronyltransferase immunoglobulin to UDP-glucuronyltransferase in intact microsomes. Intact microsomes (0.5 mg of protein) were incubated with anti-UDP-glucuronyltransferase immunoglobulin (●) or unimmoglobulin (○) and the complex was washed with Buffer A as described in Fig. 8. The suspensions of the pellets were incubated with protein A-Sepharose 4B for 4 h at room temperature. After centrifugation of the mixture at 35 x g for 5 min, the pellets were washed with Buffer A and then re-washed with Buffer A containig 1.0% sodium cholate three times. UDP-glucuronyltransferase activities toward 1-naphthol in the pellets were assayed.

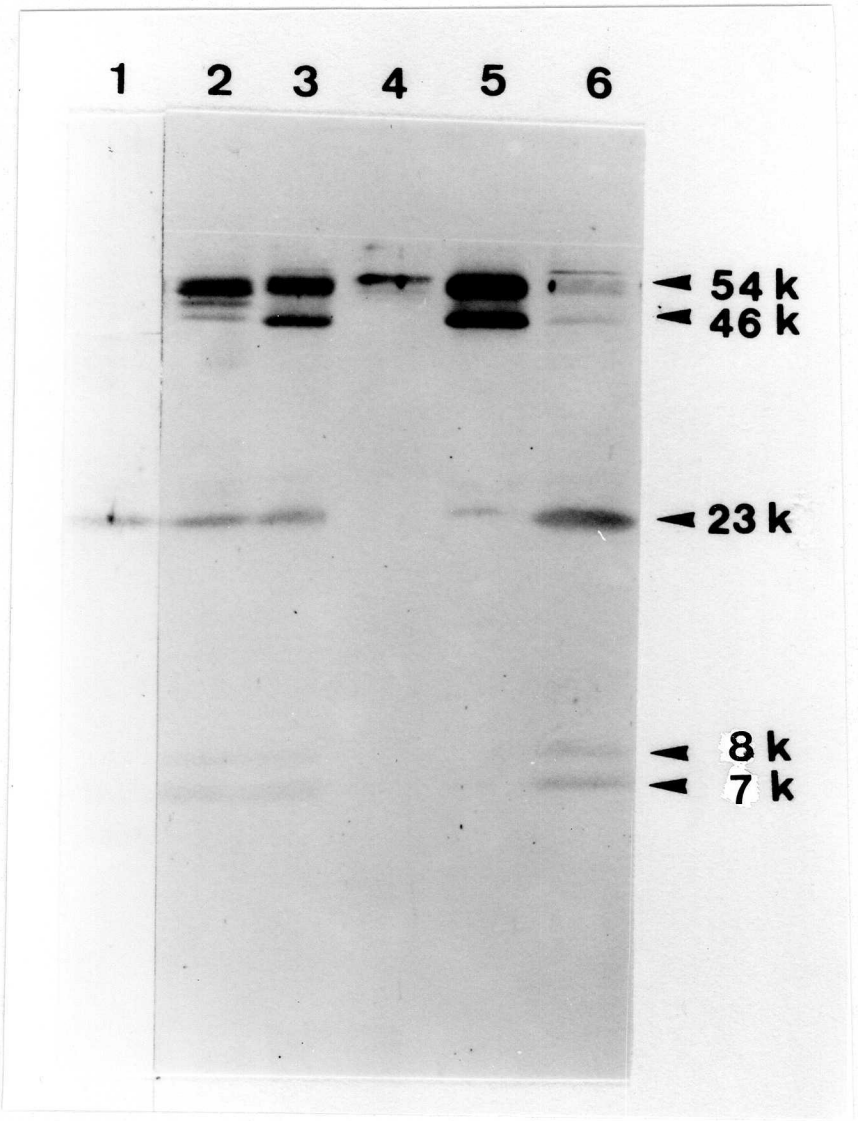

Fig. 10 Immunoblotting analysis of trypsin-digested microsomal proteins with anti-UDP-glucuronyltransferase immunoglobulin. Intact microsomes (1.0 mg of protein) in 1 ml of Buffer A were digested with 4 mg of trypsin at 4°C. After precipitation of the trypsin-digested proteins in microsomes with 1 ml of 20% trichloroacetic acid, the pellets were assayed by immunoblotting with anti-UDP immunoglobulin. Lane 1: 20 ug of trypsin only, lanes 2 and 3: microsomes (5 μg of protein) digested with trypsin for 1 or 12 h, respectively, lane 4: intact microsomes (2 μg of protein), lanes 5 and 6: pellet (10 μg of protein) and supernatant (20 μg of protein), respectively, after centrifugation of 24 h-trypsin-digested microsomes.

ACKNOWLEDGEMENTS

The author is wishes his gratitude to Professor R. Sato of the institue for Protein Research, Osaka Univercity, for his kind guidances and useful discussions. The author wishes to express his sinere thanks to Drs. Y. Imai, K. Mihara and M. Nakamura for their generous advices and many valuable suggestions. His thanks are also due to Drs. M. Komori, M. Iwasaki, T. Aoyama, M. Sakaguchi, N. Kagawa, and K. Kamitani for their useful suggestions and technical advices.

The author wishes to thank Professor A. Yuasa of the RAKUNOU GAKUEN University for his useful suggestions and continuous encouragement. He is also deeply grateful to Mr. Ohgiya and the members of his belonging laboratory for their technical supports and encouragements.