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Purification of Membrane-Bound Galactosyltransferase from Rat Liver Microsomal Fractions

By IAN H. FRASER and SAILEN MOOKERJEA
Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland A1C 5S7, Canada

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1. Rat liver microsomal preparations incubated in 1% Triton X-100 at 37°C for 1h released about 60% of the membrane-bound UDP-galactose–glycoprotein galactosyltransferase (EC 2.4.1.22) into a high-speed supernatant. The supernatant galactosyltransferase which was solubilized but not purified by this treatment had a higher molecular weight than the serum enzyme as shown by Sephadex G-100 column chromatography. 2. The galactosyltransferase present in the high-speed supernatant was purified 680-fold by an affinity-column-chromatographic technique by using a column of activated Sepharose 4B coupled with α-lactalbumin. The galactosyltransferase ran as a single band on polyacrylamide gels and contained no sialyltransferase, *N*-acetylglucosaminyltransferase or UDP-galactose pyrophosphatase activities. 3. The purified membrane enzyme had properties similar to serum galactosyltransferase. It had an absolute requirement for Mn^{2+} that could not be replaced by Ca^{2+}, Mg^{2+}, Zn^{2+} or Co^{2+}, and was active over a wide pH range (6–8) with a pH optimum of 6.5. The apparent K_m for UDP-galactose was 10.8μM. The protein α-lactalbumin modified the enzyme to a lactose synthetase by increasing substrate specificity for glucose in preference to *N*-acetylglucosamine and fetuin depleted of sialic acid and galactose. 4. The molecular weight of the membrane enzyme was 65000–70000, similar to that of the purified serum enzyme. Amino acid analyses of the two proteins were similar but not identical. 5. Sephadex G-100 column chromatography of the purified membrane enzyme showed a small peak (2–5%) of higher molecular weight than the purified serum enzyme. Inclusion of 1mM-ε-aminohexanoic acid in the isolation procedures increased this peak to as much as 30% of the total enzyme recovered. Increasing the ε-aminohexanoic acid concentration to 100mM resulted in no further increase in this high-molecular-weight fraction.

The discovery of the occurrence of glycosyltransferases in blood and tissue fluids has prompted a number of investigations of their mode of origin, clinical and physiological significance and possible function related to cell–cell interaction (Mookerjea *et al.*, 1971, 1972; Kim *et al.*, 1972*a*,*b*; Hudgin & Schachter, 1971; Swaicka, 1971; Wagner & Cynkin, 1971; Singer *et al.*, 1974; Whitehead *et al.*, 1974; Nelson *et al.*, 1973, 1974; Podolsky & Weiser, 1975*a*,*b*; Weiser *et al.*, 1976; Bhattacharya *et al.*, 1976). We have purified UDP-galactose–glycoprotein galactosyltransferase to homogeneity from rat serum, studied its kinetic properties and determined the molecular weight and amino acid composition (Fraser & Mookerjea, 1976*a*). The enzyme galactosyltransferase, in particular, has received considerable attention in recent years for its alleged role in inter- and intra-cellular functions (Shur & Roth, 1975). The activity of this enzyme in serum increases in liver disorders (Kim *et al.*, 1972*b*) and in certain neoplastic diseases (Podolsky & Weiser, 1975*b*; Weiser *et al.*, 1976; Bhattacharya *et al.*, 1976). The enzyme activity is also high in human and rat amniotic fluid (Nelson *et al.*, 1973, 1974) and in the serum of newborn rats (Jato-Rodriguez & Mookerjea, 1974). We have postulated that serum glycosyltransferases are at least partly of hepatic origin (Mookerjea *et al.*, 1972) and also obtained evidence that a 'saline-wash' fraction of the membrane-bound galactosyltransferase has a higher molecular weight than the soluble-form serum enzyme (Fraser & Mookerjea, 1976*a*). Some recent claims on the presence of an isoenzyme of galactosyltransferase in serum and increase in its activity in metastatic cancer appeared to establish some clinical significance for this enzyme (Weiser *et al.*, 1976).

We have now purified the membrane-bound galactosyltransferase from a Triton X-100 extract of rat liver microsomal preparation by affinity column chromatography and compared some of its properties with the purified soluble enzyme from

rat serum. A preliminary report of some of this work has appeared (Fraser & Mookerjea, 1976*b*).

Materials and Methods

Materials

All materials used in the experiments including the radioactive isotopes, chromatographic mediums and DSG-fetuin ('desialized' and 'degalactosylated' fetuin) were of identical origin with those described by Fraser & Mookerjea (1976*a*).

Methods

Assay of enzymes. Galactosyltransferase activity of the membrane fractions and serum was assayed by using UDP-[U-^{14}C]galactose as substrate and DSG-fetuin or monosaccharides as exogenous acceptors (Fraser & Mookerjea, 1976*a*). The methods for measuring *N*-acetylglucosaminyltransferase, sialyltransferase, UDP-galactose pyrophosphatase, protein concentration of the enzyme samples and amino acid composition of the purified enzymes have been described previously (Fraser & Mookerjea, 1976*a*).

Preparation of microsomal fraction and 'Triton X-100 supernatant'. A microsomal fraction was prepared from rat liver by the method of Mookerjea (1972). The microsomal pellets were suspended in 0.25 M-sucrose and a Tris/HCl buffer (35 mM, pH 7.5) containing 25 mM-KCl and 5 mM-$MgCl_2$ (TKM buffer) and 1% (v/v) Triton X-100. After incubation at 37°C for 1 h the suspension was centrifuged at 100000*g* in a Spinco model L ultracentrifuge for 1 h. The supernatant was drawn off with a Pasteur pipette and designated the 'Triton X-100 supernatant'.

Purification of liver microsomal galactosyltransferases. Galactosyltransferase was isolated and purified by using a column (2 cm × 5 cm) of Sepharose–α-lactalbumin as described before (Andrews, 1970; Fraser & Mookerjea, 1976*a*) except that buffer contained 1% Triton X-100. About 8.2 mg of α-lactalbumin was bound to 1 g of CNBr-activated Sepharose 4B. The binding was monitored by reading transmission at 280 nm of the α-lactalbumin before and after incubation with Sepharose 4B. Sucrose was removed from the Triton X-100 supernatant by dialysis against the column buffer lacking Triton X-100 or *N*-acetylglucosamine. The dialysed supernatant containing 1% Triton X-100 and 3 mM-*N*-acetylglucosamine was applied to the column for purification.

Analytical polyacrylamide-gel electrophoresis. Polyacrylamide gels were run as described before (Fraser & Mookerjea, 1976*a*). For molecular-weight determination, SDS*/polyacrylamide-gel electrophoresis was carried out as described by Neville (1971) and Weber & Osborn (1969).

Column chromatography. Columns (2 cm × 21 cm) of Sephadex G-100 were equilibrated in 0.1 M-Tris/HCl buffer, pH 7.5, containing 0.1 M-NaCl, 0.02% NaN_3 and 1% Triton X-100 and were run with descending flow by the method of Killander (1964).

A column (2 cm × 21 cm) of Bio-Gel P-150 was equilibrated in the same buffer as above but without Triton X-100.

* Abbreviation: SDS, sodium dodecyl sulphate.

Results

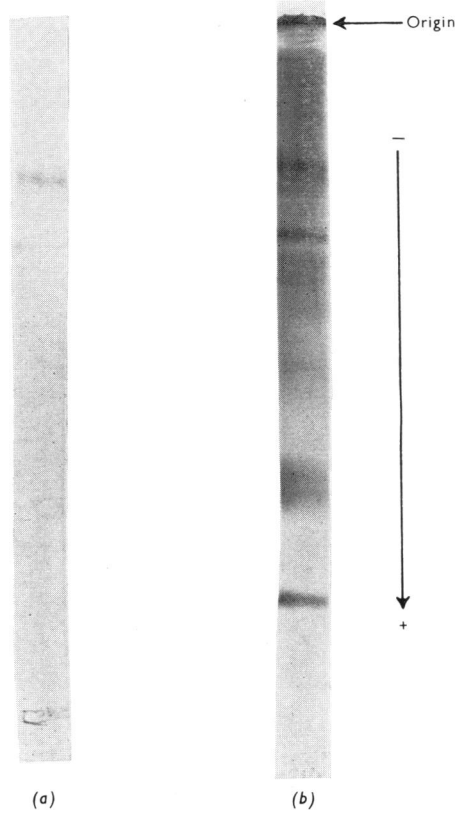
Comparison of serum and Triton X-100 supernatant enzyme by Sephadex chromatography

Triton X-100 (0.5%) stimulated membrane-bound microsomal galactosyltransferase as much as 15-fold. About 60% of this membrane-bound enzyme is extracted into the 100000*g* supernatant after incubation with 1% Triton X-100 (Table 1). Since the specific activity of the enzyme in the Triton X-100 extract is similar to that of the pellet, the galactosyltransferase appears to be solubilized non-specifically along with other proteins by this treatment. On Sephadex G-100 column chromatography the galactosyltransferase activity ran as a distinct peak which was ahead of and well separated from serum galactosyltransferase (Fig. 1). When serum and Triton X-100 supernatants were combined and chromatographed on Sephadex G-100 columns as shown for Fig. 1, the galactosyltransferase peaks remained distinct and well separated (results not shown).

Table 1. *Galactosyltransferase activity of microsomal samples*

The enzyme assay is described in the Materials and Methods section. Microsomal preparations in the presence of 1.0% Triton X-100 were incubated at 37°C for 60 min and a sample was taken for assay. The rest was centrifuged at 100000*g* for 60 min and the supernatant and pellet (made up to starting volume in buffer) were also tested for galactosyltransferase activity.

Fractions	Protein (μg/assay)	Galactosyltransferase activity (pmol/h)	Galactosyltransferase activity (nmol/h per mg of protein)
Microsomal preparation + Triton X-100	119	655	5.50
Triton X-100 supernatant	77	394	5.10
Microsomal pellet	54	275	5.10



EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis (*pH* 8.3) *of purified rat liver microsomal galactosyltransferase*

The disc gel electrophoresis method is described in the Materials and Methods section. (*a*) Purified microsomal enzyme; (*b*) Triton X-100 supernatant.

Purification of membrane-bound enzyme by affinity column chromatography

The galactosyltransferase present in the Triton X-100 supernatant was purified by the affinity-chromatography technique whereby α-lactalbumin was coupled to CNBr-activated Sepharose 4B. About 70% of the total enzyme activity was retained on such a column in the presence of 3 mM-*N*-acetylglucosamine and was released from the column when *N*-acetylglucosamine was omitted from the elution buffer (Fig. 2). It is important to emphasize here that Triton X-100 (1%) was required in the elution buffer for the enzyme to remain active. Washing the column with 0.1% Triton X-100 and eluting the enzyme with 0.1% Triton X-100 resulted in a virtual loss of enzyme activity.

It is also noteworthy that a clear-cut profile of the elution of the enzyme by buffer lacking *N*-acetylglucosamine was achieved (Fig. 2) only when sucrose was adequately removed from the Triton X-100 supernatant. Sucrose probably interferes with the tight binding of the enzyme to α-lactalbumin in the presence of *N*-acetylglucosamine. After rechromatography of the eluate, a pure enzyme fraction was obtained, which gave essentially a single band on analytical polyacrylamide-gel electrophoresis (Plate 1). The membrane enzyme as obtained is probably 90% pure, and shows some trace contaminating bands. Despite minor contamination, both the serum (Fraser & Mookerjea, 1976*a*) and membrane enzymes have remarkably similar specific activity and amino acid composition. The purified enzyme contained little or no sialyltransferase, *N*-acetylglucosaminyltransferase or UDP-galactose pyrophosphatase activity (Table 2). In a pure form the enzyme was very unstable and could not be stored frozen without loss of activity. Removal of Triton X-100 from the pure enzyme by stirring the solution with SM-2 beads resulted in considerable loss of activity. However, this treatment resulted in little loss of protein, as was also noted by Holloway (1973). By using affinity chromatography the galactosyltransferase was purified 680-fold from the microsomal preparation with a recovery of about 30–40%. The purified membrane enzyme has a similar specific activity to that reported (Fraser & Mookerjea, 1976*a*) for the pure serum galactosyltransferase (3750 versus 3960 μmol/h per mg of protein).

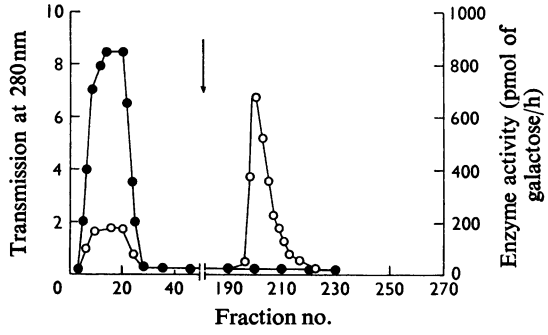


Fig. 2. *Effect of withdrawal of N-acetylglucosamine on the elution of Triton X-100 supernatant galactosyltransferase from a α-lactalbumin–Sepharose 4B column*

α-Lactalbumin was coupled to a CNBr-activated Sepharose 4B column and the enzyme was retained on the column owing to its affinity for α-lactalbumin in the presence of 3 mM-*N*-acetylglucosamine. The enzyme was released from the column when *N*-acetylglucosamine was withdrawn from the buffer (shown by the arrow). The eluate fractions (2.5 ml) were analysed for protein (●) (transmission at 280 nm) and the enzyme activity (○) was assayed as described in the Materials and Methods section.

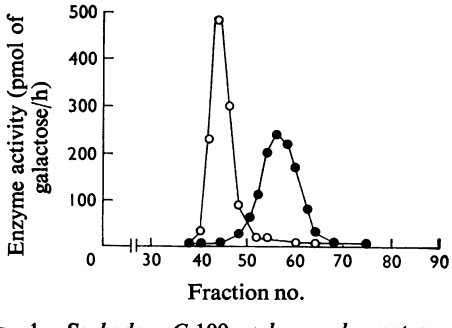


Fig. 1. *Sephadex G-100 column chromatography of galactosyltransferase from serum and Triton X-100 supernatant*

A column (2.0 cm × 21 cm) of Sephadex G-100 was equilibrated in 0.1 M-Tris/HCl, pH 7.5, containing 0.1 M-NaCl, 1.0% Triton X-100 and 0.02% NaN_3. A 1.0 ml sample of serum or Triton X-100 supernatant was applied to the top of the column and eluted by a descending flow of column buffer. The eluate fractions (0.44 ml/fraction) were assayed for enzyme activities as described in the Materials and Methods section: serum (●), Triton X-100 supernatant (○).

The amino acid composition of the pure enzyme is shown in Table 3. Serine content is high, whereas the apolar amino acid content is low.

Properties of the pure microsomal galactosyltransferase

The purified membrane galactosyltransferase showed an absolute requirement for Mn^{2+} which could not be replaced by Mg^{2+}, Ca^{2+}, Zn^{2+} or Co^{2+} (Table 4). The enzyme activity was maximal at

Table 2. *Enzyme activities of liver microsomal fractions*

The assay for measuring enzyme activities is described in the Materials and Methods section. Rat liver microsomal galactosyltransferase was purified by rechromatography on Sepharose 4B–α-lactalbumin. Tr, trace.

Fraction	Glycoprotein glycosyltransferase activity (pmol/h per mg of protein)			UDP-galactose pyrophosphatase (nmol of galactose 1-phosphate formed/h per mg of protein)
	Galactosyltransferase	Sialyltransferase	*N*-Acetylglucosaminyltransferase	
Triton X-100 supernatant	3700	1300	145	56.5
Purified enzyme	3750×10^3	Tr*	Tr*	Tr*

* The purified liver fraction had less than 1.0% of the original activity found in the microsomal preparation.

Table 3. *Amino acid composition of rat serum and liver microsomal galactosyltransferase*

Amino acid analyses were run on a Beckman 120C amino acid analyser after digestion of the enzyme in 6M-HCl for 20h under vacuum. Duplicate analyses of a separate enzyme preparation showed essentially similar composition. N.D., Not determined. The number of residues per molecule is based on a molecular weight of 68000, minus 12% for carbohydrate.

Amino acid	Calculated no. of residues per molecule	
	Serum	Microsomal
Lysine	51	46
Histidine	11	15
Arginine	4	8
Aspartic acid	35	36
Threonine	21	21
Serine	135	137
Glutamic acid	76	83
Proline	14	15
Glycine	106	92
Alanine	42	39
Half-cystine	N.D.	N.D.
Valine	14	15
Methionine	N.D.	N.D.
Isoleucine	9	10
Leucine	13	15
Tyrosine	7	6
Phenylalanine	7	8
Tryptophan	N.D.	N.D.

12.5mM-Mn^{2+}. Higher concentrations up to 50mM-Mn^{2+} led to a slight decrease in enzyme activity; EDTA at 12mM inhibited the enzyme activity by 95% in the presence of Mn^{2+}. For the assay of enzyme in the Triton X-100 supernatant, ATP (2mM) was required for inhibition of UDP-galactose pyrophosphatase activity. The absence of pyrophosphatase from the pure enzyme fraction is further suggested by the fact that ATP is not required for assaying the pure enzyme (Table 4). The pH profile for the enzyme is wide, with a maximum at 6.5 (Fig. 3). The galactosyltransferase activity was proportional to time

Table 4. *Properties of purified rat liver microsomal galactosyltransferase*

Enzyme activity was measured as described in the Materials and Methods section; 25μl of enzyme solution, purified by rechromatography on an α-lactalbumin–Sepharose 4B column which contained approx. 48ng of protein, was used in each assay.

Assay mixture	Enzyme activity (pmol/h)
Complete	184
Minus enzyme	5
Minus DSG-fetuin	3
Minus Mn^{2+}	38
Minus ATP	179
Minus ATP plus EDTA (12mM)	30
Minus ATP plus Mg^{2+} (12.5mM)	32
Minus ATP plus Cu^{2+} (12.5mM)	21
Minus ATP plus Zn^{2+} (12.5mM)	7
Minus ATP plus Co^{2+} (12.5mM)	58
Minus ATP plus bovine serum albumin (25μg)	189

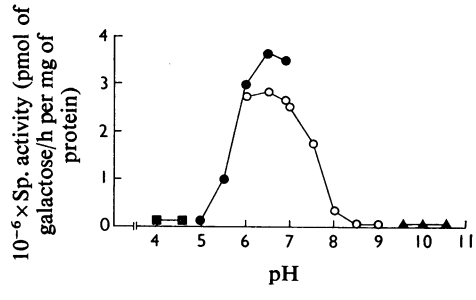


Fig. 3. *Effect of pH on activity of purified membrane galactosyltransferase*

Buffers (125mM) used for the assay were sodium acetate/acetic acid, pH4.0–4.6 (■); Mes (4-morpholine-ethanesulphonic acid)/NaOH, pH5.0–6.8 (●); Tris/HCl, pH6.0–9.0 (○); and glycine/NaOH, pH9.6–10.6 (▲). The enzyme assay method is described in the Materials and Methods section.

up to 1h and with enzyme concentration from 20 to 100ng of protein. The apparent K_m of the pure enzyme for UDP-galactose is 10.8μM (Fig.4). This is similar to the value reported for purified rat serum galactosyltransferase (Fraser & Mookerjea, 1976*a*). Fig. 5 shows the enzyme activity towards DSG-fetuin, *N*-acetylglucosamine and glucose as acceptors in the absence or in the presence of α-lactalbumin. Addition of α-lactalbumin modified the enzyme such that it made lactose from UDP-galactose and glucose and inhibited its ability to synthesize *N*-acetyl-lactosamine. This enzyme is quite similar to lactose synthetase A from bovine milk (Brew *et al.*, 1968). Further, the α-lactalbumin also inhibited the ability of DSG-fetuin to act as acceptor. This inhibition was shown when the enzyme was assayed by two different methods. For the assay of *N*-acetyl-lactosamine synthetase activity, the pure enzyme showed a requirement for albumin (125μg/assay) as a stabilizer for the enzyme. Without this addition *N*-acetyl-lactosamine synthetase activity was negligible. Pure serum enzyme showed an identical requirement for albumin (Fraser & Mookerjea, 1976*a*). For assaying the enzyme with DSG-fetuin and glucose as acceptors this addition was not necessary.

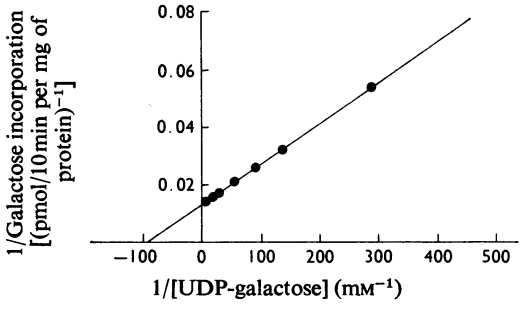


Fig. 4. *Effect of different UDP-galactose concentrations on the activity of purified membrane galactosyltransferase*
The enzyme assay method is described in the Materials and Methods section. The data were plotted by the method of Lineweaver & Burk (1934). The apparent K_m value of the enzyme for the substrate is 10.8μM.

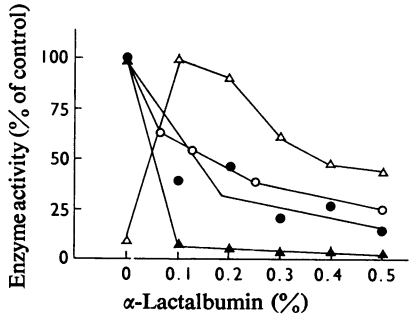


Fig. 5. *Effect of α-lactalbumin on the pure membrane enzyme to transfer galactose to DSG-fetuin, glucose and N-acetylglucosamine*
Enzyme was assayed by the method of high-voltage electrophoresis as described in the Materials and Methods section, except that for *N*-acetyl-lactosamine synthetase activity the pure enzyme showed an absolute requirement for albumin (125μg/assay) as a stabilizer. Transfer of galactose from UDP-galactose to glucose (△), *N*-acetylglucosamine (▲) and DSG-fetuin (●) is shown as a function of the concentration of α-lactalbumin. In one experiment with DSG-fetuin as acceptor (○), the galactosyltransferase activity was measured by acid precipitation and filtration as described in the Materials and Methods section.

Comparison of pure serum and liver galactosyltransferase

Both enzymes gave a similar pattern of SDS/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) and on polyacrylamide-gel electrophoresis at pH8.3. The molecular weights in each case were between 65000 and 70000. We have previously reported (Fraser & Mookerjea, 1976*a*) that the pure serum enzyme had a molecular weight of 43000 as measured on SDS/polyacrylamide gels (Neville, 1971). However, on SDS/polyacrylamide gels pre-

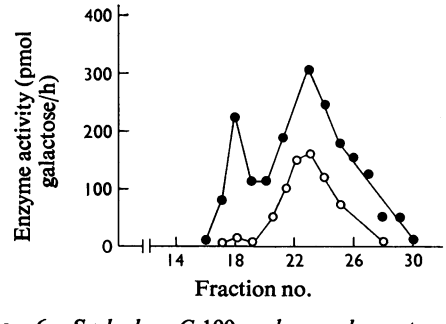


Fig. 6. *Sephadex G-100 column chromatography of purified liver microsomal galactosyltransferase*
A column (2.0cm×21cm) of Sephadex G-100 was equilibrated in 0.01M-Tris/HCl, pH7.5, containing 0.04M-KCl, 1.0% Triton X-100, 0.02% NaN_3 and 2mM-β-mercaptoethanol. A 1.0ml sample of pure microsomal enzyme (○) was applied to the column and eluted by a descending flow of buffer. The column was equilibrated in the same buffer containing 1mM-ε-aminohexanoic acid and 1.0ml of microsomal enzyme (●) isolated in the presence of 1mM-ε-aminohexanoic acid was applied to the column. Each eluted fraction contained 1.0ml.

pared by the method of Weber & Osborn (1969) the molecular weight of the pure serum enzyme was 65000–70000. Further, as the serum enzyme had a molecular weight of 63000 on a Bio-Gel P-150 column when compared with various standards (results not shown), we consider the latter observation the more reliable.

On Sephadex G-100 column chromatography, most of the microsomal enzyme was eluted at a position slightly ahead of the serum enzyme. A small portion (2–5%) appeared to be of a higher molecular weight as it was eluted first (Fig. 6). When 1 mM-ε-aminohexanoic acid was included in all the isolation procedures this peak increased to as much as 30% of the total enzyme (Fig. 6). Increasing the ε-amino hexanoic acid concentration to 100mM resulted in no further increase in the higher-molecular-weight fraction.

The amino acid content (Table 3) of the microsomal enzyme was similar to but not identical with that of the serum galactosyltransferase. Both had a high serine and low leucine and isoleucine content. However, the membrane enzyme had more histidine, arginine and glutamic acid and less lysine and glycine. In general both enzymes showed similar kinetic properties.

Discussion

The preparation of purified glycosyltransferases appears to be a prerequisite for further advances in our understanding of the physiological function of this group of enzymes in mammalian systems. The question of the relationship between the membrane-bound and soluble forms of these enzymes could be also resolved by a study of the purified enzymes. In the present work, we have isolated a purified membrane-bound galactosyltransferase from rat liver microsomal fraction. The enzyme activity derived from Triton X-100 extracts of membranes was shown to be of higher molecular weight by Sephadex-gel chromatography when compared with the enzyme purified from rat serum. Because the liver enzyme is membrane-bound, whereas the serum enzyme is soluble, there should be some differences between the two proteins. Such differences have been reported for bovine galactosyltransferase from colostrum and milk (Magee *et al.*, 1974). The requirement for Triton X-100 or lysophosphatidylcholine for the activation of the membrane-bound enzyme also emphasizes its difference from the soluble enzyme. In the purification procedure for rat serum galactosyltransferase, inclusion of a small amount of Triton X-100 (0.1%) in the elution fluids was essential for recovering the enzyme in active form (Fraser & Mookerjea, 1976*a*). This suggested that the activity site of the enzyme protein requires a detergent for stabilization. It is noteworthy that a 10-fold larger concentration of Triton X-100 (1%) in the eluent was required for the isolation of pure membrane-bound enzyme. The membrane enzyme is probably aggregated much more easily, which is reflected by its early exclusion from the Sephadex gels. Also, the membrane-enzyme showed a requirement for a lipid-micro-environment to remain active. Triton X-100 or lysophosphatidylcholine appears to provide this environment when the enzyme was being purified. Higher amounts of Triton X-100 in the purification procedure of the membrane enzyme are therefore most likely to have two functions, i.e. providing a proper lipid micro-environment and stabilizing the active site.

The kinetic properties of the pure membrane and soluble enzymes are strikingly similar. This also supports the idea that the serum enzyme originates, at least partly, from the liver. In studies on the effect of α-lactalbumin on the lactose synthetase activity, it was observed that increasing doses of α-lactalbumin inhibited the pure membrane enzyme when DSG-fetuin was used as an acceptor. This inhibition was not observed in similar studies with pure serum enzyme (Fraser & Mookerjea, 1976*a*). The significance of this difference is at present not understood. The molecular-weight determinations on SDS/polyacrylamide gels and on Bio-Gel P-150 columns showed hardly any difference between the pure membrane and serum enzymes. The amino acid compositions of the pure membrane and serum enzymes also showed only minor differences. This may suggest that the differences between the two enzyme proteins are indeed very small. It is, however, possible that the membrane galactosyltransferases may become degraded during the isolation procedures (Magee *et al.*, 1976). The purified enzyme is probably more vulnerable to the action of small amounts of proteinases likely to be present. This possibility was examined by the inclusion of ε-aminohexanoic acid, an inhibitor of plasmin activity (Magee *et al.*, 1976), in all extraction and elution fluids. A small peak of a higher-molecular-weight form of the pure enzyme was eluted from the Sephadex G-100 column and increased to as much as 30% in the presence of 1 mM-ε-aminohexanoic acid. Further analysis of the proteins present in this peak may reveal more important differences between the pure membrane-derived and serum enzymes.

Recent observations of the presence of a higher-molecular-weight form of galactosyltransferase in the serum of patients with metastatic carcinoma (Weiser *et al.*, 1976) have generated considerable interest in the clinical significance of this group of enzymes present in circulation. Further studies on this alleged isoenzyme, its purification and kinetic properties can be expected to shed new light on the role of this enzyme in malignant disorders. However, it is also possible that a higher-molecular-

weight form of the enzyme normally present in the membrane in bound form may lose its binding property and be released in increasing amounts into the serum as a result of malignancy of certain tissues. In that case, a close examination of the differences and similarities between the membrane-bound and soluble forms of glycosyltransferases will prove to be a rewarding area of study in future.

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