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Responses of Microsomal UDP-Glucuronyltransferase to Trypsin

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SUMMARY

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The UDP-glucuronyltransferases of rat and guinea pig liver microsomal fractions prepared in 154 mM KCl responded very differently to proteolysis of the membrane surface by trypsin. Guinea pig transferase was inactivated biphasically: a rapid inactivation stage was followed by a much slower one. The slow inactivation was accelerated by chymotrypsin and the combined effects of the two proteases caused almost total loss of activity. A large part of the activity lost on trypsin treatment was recovered on adding phosphatidylcholine. Rat transferase, on the other hand, was much more resistant to proteolysis. Trypsin elicited a triphasic response which occurred on a long time scale. Firstly, a small, rapid inactivation was observed, followed by a slow activation, then finally, inactivation. However, when areas of the microsomal membranes shielded from proteolysis in intact preparations were made accessible to trypsin by adding Triton X-100, the UDP-glucuronyltransferases of both animals were rapidly inactivated in similar fashion. These results may be explained if UDP-glucuronyltransferase molecules are located differently in KCl-prepared microsomal membranes of the two species. In guinea pigs the enzyme molecules probably are situated at or near the membrane surface, while in rats the majority appear to occupy sites deep within the membrane.

INTRODUCTION

Studies of the effects of membrane perturbants on UDP-glucuronyltransferase (EC 2.4.1.17) in liver microsomal preparations have shown that the enzyme's activity is modulated by structural components of the vesicular membrane. A highly pure transferase preparation, free from other membrane constituents, is not available yet, and our knowledge of membrane control of glucuronidation has come from probing the enzyme and its hydrophobic environment with reagents which perturb membrane structure (1–4).

Membrane perturbants have striking effects on UDP-glucuronyltransferase activity, but apparently conflicting results have been reported. The most pronounced effect of some perturbants in certain microsomal preparations was a strong enzyme activation (5–18). For example, when microsomal fractions were prepared from rat or guinea pig liver using 0.25 M sucrose, transferase activities were very low and treatment with phospholipase A (EC 3.1.1.4) or detergents greatly increased activity (14). Thus, the predominant effect of the membrane on the enzyme in sucrose-prepared microsomal fractions is a powerful restraint on catalytic activity from which it is released by disrupting the membranes with low concentrations of detergents (5, 10, 14, 18) or brief treatments with phospholipases (10, 12, 14,

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18). Low levels of lipid peroxidation (15) and mechanical methods (10, 17, 18) have similar effects. However, when membranes from the two species were prepared and washed in 154 mM KCl the transferase, as well as being much more active, responded differently to membrane perturbation. Rat liver UDP-glucuronyltransferase in these preparations was still activated by phospholipases (9, 14, 16) and detergents (6–9, 11, 13, 14, 16) but the degree of latency was much less than in sucrose-prepared membranes. In contrast, the guinea pig enzyme was now in an extremely active form which could not be activated significantly by perturbation (13, 14, 19, 20). Thus, the KCl-isolation method appears to have released the guinea pig transferase from latency almost completely. Moreover, the transferase in these preparations was inactivated when the membrane phospholipids were degraded with phospholipase A (19–21) and it has been shown that this fully active enzyme form is phospholipid-dependent (22). It was concluded that the microsomal membrane provided a suitable phospholipid environment to support full enzyme activity. Thus the microsomal membrane can have two opposing effects on UDP-glucuronyltransferase. It can both restrict and maintain activity, and in some microsomal preparations these effects are superimposed (9, 10, 12–16, 18). Response to membrane perturbants is the net result of interfering with both these forms of modulation. In many types of microsomal preparation release from latency obscures any effect on the activity-maintaining function (18, 23), but even in these preparations extensive degradation of phospholipids by prolonged exposure to phospholipases (9, 10, 12, 14) and peroxidizing agents (15) also inactivated the transferase.

Recent work favors the view that latency of UDP-glucuronyltransferase activity is due to the location of enzyme molecules within the membrane; permeation of substrates to the sites of reaction is hindered by a lipophilic membrane barrier (4, 6–8, 15–17). This permeability hypothesis implies that in rat microsomal fractions prepared in KCl, UDP-glucuronyltransferase molecules are located deep within the microsomal membrane, while in the corresponding guinea pig preparations enzyme activity is not restricted by a permeability barrier, possibly because its molecules are located differently in the membrane, i.e., at or near its outer surface.

The dispositions of proteins within membranes have been investigated by several techniques (24) including controlled proteolysis. Degradation of microsomal membranes by proteases appears to be restricted to proteins located near their outer surface (25–31). If there are differences in location of UDP-glucuronyltransferase molecules in these microsomal membranes from rat and guinea pig, its activity in the two species is expected to respond differently to proteolysis. We have therefore examined the effects of trypsin (EC 3.4.4.4) on UDP-glucuronyltransferase activity in these preparations to establish if responses to this membrane perturbant are consistent with a difference in location of enzyme molecules within the microsomal membranes. Since preparation of liver microsomal membranes in 154 mM KCl affords sources of both latent (rat) and fully active enzyme (guinea pig), this isolation method has been used throughout. A preliminary account of part of this work has been published (32).

MATERIALS AND METHODS

Trypsin (type 1, twice crystallized, free of phospholipase A activity), soybean trypsin inhibitor (type 1-S) and chymotrypsin (type 1, three times crystallized) were from Sigma London Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K., and used as freshly prepared solutions in H_2O.

Male guinea pigs (Hartley, 250–450 g) were from Fisons Pharmaceuticals, Loughborough, Leicestershire, U.K., or from David Hall, Newchurch, Burton-on-Trent, Staffordshire, U.K. Male rats (Wistar, 150–250 g) were from Fisons or from Animal Supply Ltd., Welwyn, Hertfordshire, U.K. The animals were starved overnight, killed and liver microsomal fractions prepared and washed in 154 mM KCl (14). The microsomal pellets were suspended in KCl solution and adjusted to a concentration of 20 mg of protein/ml, measured with a biuret reagent (33). Rat microsomal fractions

were stored at 0° and used within 1 hr of preparation; those from guinea pigs were stored at −20° and used within 48 hr. These different storage conditions have been shown to have little effect on either the UDP-glucuronyltransferase activity of microsomal fractions or the responses of the enzymes to membrane disruption with detergents (13, 14), and thus are assumed not to affect the changes in transferase activity caused by trypsin reported here.

Microsomal membranes (final conc. 5 mg of protein/ml, except where stated otherwise) were digested with protease (50 μg/mg of microsomal protein, except where stated otherwise) by shaking at 30° in a medium containing 20 mM Tris-HCl buffer, pH 7.5. Trypsin activity was terminated by adding a twofold excess by weight of trypsin inhibitor.

Other microsomal fractions (final conc. 5 or 10 mg of protein/ml) were treated with Triton X-100 (0.1%), sodium deoxycholate (0.06%) or digitonin (0.6%) for 10 min at room temperature in presence of 12.5 mM Tris-HCl buffer, pH 8.0.

Phosphatidylcholine was purified and dispersed in 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and its concentration determined as P content after drying (20).

Since the response of UDP-glucuronyltransferase to membrane perturbants varies slightly with the concentration of UDP-glucuronate used to determine its activity (14), enzyme activities (forward reaction) were measured at both high and low substrate concentrations. At the lower concentrations with guinea pig microsomal fractions, activities were measured with 0.17 mM *p*-nitrophenol and 0.167 mM UDP-glucuronate (13). At this concentration of sugar nucleotide the transferase activities of rat microsomal fractions are very low and their activities were determined with 0.17 mM *p*-nitrophenol and 0.5 mM UDP-glucuronate (13). At the higher substrate concentrations (0.6 mM *p*-nitrophenol and 4 mM UDP-glucuronate), our previous method (14) was used but with the following improvements. The incubation volume during assay was increased to 0.8 ml and, at seven appropriate times, 0.1 ml portions were removed and added to 2 ml of cold 0.1 M trichloroacetic acid. Enzyme activities are quoted as n mol of *p*-nitrophenol glucuronidated per min/mg of microsomal protein.

The activity of the UDP-glucuronyltransferase reverse reaction was measured by a method based on those of Zakim and Vessey (34) and Berry and co-workers (17). Reaction mixtures (final vol. 0.5 ml) contained 50 mM KH_2PO_4-KOH buffer, pH 7.1, 4 mM EDTA, 10 mM glucaro-1,4-lactone, 4 mM *p*-nitrophenylglucuronide, 2 mM UDP and microsomal protein. Incubation with shaking was at 37°, and at four appropriate times, 0.1 ml samples were withdrawn and added to 2 ml of cold 0.1 M trichloroacetic acid. After centrifuging, 10 M KOH (20 μl) was added to 1 ml portions of the supernatant solutions and the absorbances determined at 400 nm. With this system no *p*-nitrophenol was liberated in the absence of UDP using native or detergent-treated membranes. Activities (nmol of *p*-nitrophenol produced per min/mg of microsomal protein) were calculated from initial rates and never more than 10% of the glucuronide was transformed.

Nucleoside diphosphatase activity was determined using a reaction medium (final vol. 1 ml) containing 50 mM Tris-HCl buffer, pH 7.1, 4 mM UDP and microsomal protein. Incubation with shaking was at 37° for 10 min and the reaction was stopped by adding cold 1 M $HClO_4$ (1 ml). After centrifuging, 0.5 ml portions of the supernatant fraction were assayed for the inorganic phosphate released (35). Controls were stored on ice and were of identical composition but with the microsomal fractions added after the $HClO_4$.

RESULTS AND DISCUSSION

The initial experiments used guinea pig UDP-glucuronyltransferase assayed at low substrate concentrations. When microsomal suspensions were digested for 30 min with increasing concentrations of trypsin, the transferase was progressively inactivated up to about 40 μg of protease/mg of microsomal protein (Fig. 1a). Above this concentration very little further inactivation was observed. This finding was con-

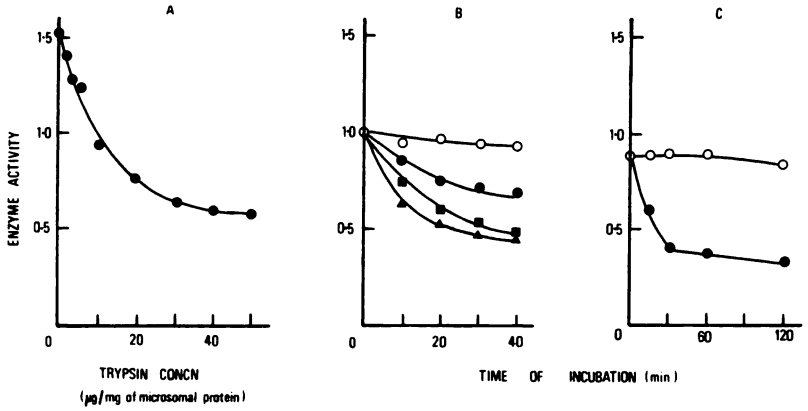

FIG. 1. *Effect of trypsin treatment on the UDP-glucuronyltransferase activity (assayed at low substrate concentrations) of guinea pig microsomal membranes*

(A) Microsomal suspensions were digested with trypsin at the concentrations indicated for 30 min. Trypsin inhibitor was added to the digests and portions assayed for transferase activity. (B) Microsomal suspensions were incubated without trypsin (○) or with trypsin at the following concentrations, 5 (●), 40 (■) and 50 (▲) μg/mg of microsomal protein. Portions of the digests were withdrawn after various times and assayed for transferase activity in presence of trypsin inhibitor. (C) Microsomal suspensions were incubated with 50 μg of trypsin/mg of microsomal protein (●) or without the protease (○). Portions of the digest were withdrawn after various times and assayed for transferase activity in presence of trypsin inhibitor.

firmed by the results of Fig. 1b, and a trypsin concentration of 50 μg/mg of microsomal protein was selected for subsequent work. Figure 1c shows that no inactivation of the transferase occurred in the absence of trypsin even after incubating at 30° for 120 min, and suggests that about 60–70% of UDP-glucuronyltransferase activity was rapidly lost and that the remainder was relatively resistant, being inactivated only very slowly even when proteolysis was allowed to continue for 120 min.

Trypsin is known to catalyze its own hydrolytic inactivation (36) and, to ensure that this was not responsible for the incomplete transferase inactivation, fresh trypsin was added to a digest once the slow inactivation stage was reached. Figure 2a shows that the rate of inactivation of guinea pig UDP-glucuronyltransferase did not increase after this addition. However, when chymotrypsin (EC 3.4.4.5) was added to a digest at the start of the slow decline phase, the "trypsin-resistant" transferase was rapidly inactivated (Fig. 2b). These results suggest that the decline in the rate of UDP-glucuronyltransferase inactivation by trypsin is due to depletion of susceptible peptide bonds.

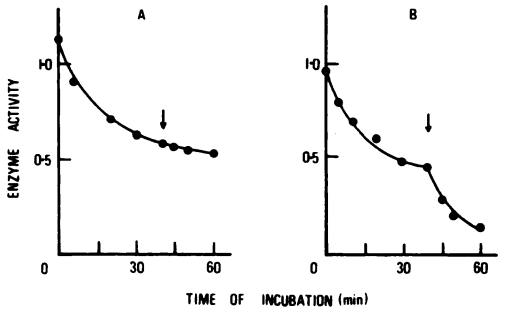

FIG. 2. *Effect of additional trypsin or chymotrypsin on the slow stage of guinea pig microsomal UDP-glucuronyltransferase inactivation by trypsin*

Microsomal suspensions were digested with trypsin. After 40 min, (A) fresh trypsin was added to bring its total concentration to 100 μg/mg of microsomal protein, or (B) chymotrypsin was added to a concentration of 50 μg/mg of microsomal protein. Portions of the digests were withdrawn after various times and transferase activity determined, at low substrate concentrations, in presence of trypsin inhibitor. Chymotrypsin activity was not inhibited during transferase assays.

UDP-glucuronyltransferases in intact microsomal preparations yield biphasic double-reciprocal kinetic plots with a distinct discontinuity near 0.8 mM UDP-glucuronate (37), and our earlier work (14)

revealed slight differences in their responses to phospholipid modification by phospholipase A when different concentrations of substrates were used in transferase assays. Therefore, the experiment of Fig. 1c was repeated, UDP-glucuronyltransferase activities being measured with 0.6 mM *p*-nitrophenol and 4 mM UDP-glucuronate. Figure 3 shows that essentially the same result was obtained after assaying at these higher substrate concentrations; trypsin caused rapid loss of about 60% of the transferase activity of native membranes and the remainder declined very slowly.

These results are consistent with the observations of Mowat and Arias (21) on the effect of trypsin on the UDP-glucuronyltransferase of guinea pig liver microsomal membranes prepared in a KCl medium. They are capable of a number of interpretations, two of which are most obvious. (1) Trypsin hydrolyzes and thus reduces the catalytic activity of all or most of the transferase molecules, a substantial fraction of which must therefore be located at or near the outer surface of the microsomal membrane. (2) The loss of transferase activity is due not to hydrolysis of enzyme protein but to hydrolysis of other membrane proteins located at or near the outer membrane surface, an interpretation implying that these proteins are required for full transferase activity. Association between the two need not be a direct one; these membrane proteins might be required for association between transferase and essential phospholipid or for enzyme-substrate interactions.

When UDP-glucuronyltransferase activity was measured at the lower substrate concentrations, micellar dispersions of phosphatidylcholine restored almost full activity to membranes whose activity had been reduced by about two-thirds by trypsin (Fig. 4). When the higher substrate concentrations were used to assay activity, phosphatidylcholine caused partial reactivation. This reversibility of protease inactivation suggests that any hydrolysis of transferase molecules must be so limited as not to destroy the enzyme's active site. Reversal of inactivation by phosphatidylcholine is not unlike the reactivation of guinea pig UDP-glucuronyltransferase by the phospholipid after inactivation by phospholipase A (20), and it is possible that the alterations of membrane structure by either

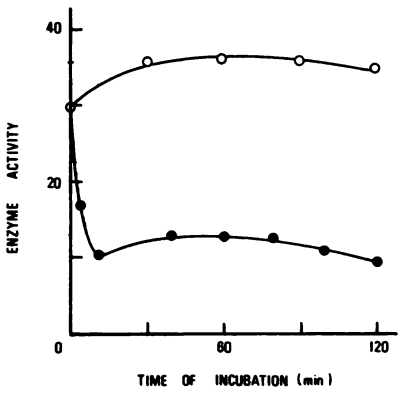

FIG. 3. *Effect of trypsin treatment on the UDP-glucuronyltransferase activity (assayed at high substrate concentrations) of guinea pig microsomal membranes*

Microsomal suspensions were digested with (●) and without (○) trypsin. After various times, portions of the digest were withdrawn and trypsin inhibitor added. Portions of these mixtures were then assayed for transferase activity.

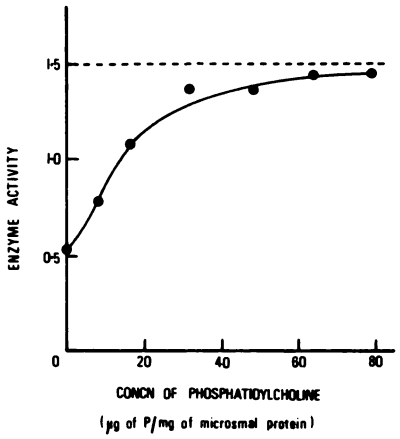

FIG. 4. *Effect of phosphatidylcholine micelles on the UDP-glucuronyltransferase activity of trypsin-treated guinea pig microsomal membranes*

A microsomal suspension (10 mg of protein/ml) was digested with trypsin for 60 min. Trypsin inhibitor was added and portions of the digest added to dispersions of phosphatidylcholine at the concentrations indicated. After 5 min at room temperature, samples of these mixtures were assayed for transferase activity at low substrate concentrations. The dashed line indicates the transferase activity of untreated microsomal membranes.

the phospholipase or trypsin cause transferase molecules to adopt conformations of greatly reduced activity which can be converted into more reactive, if not their original forms by phosphatidylcholine. These results alone do not provide evidence for the location of transferase molecules within the membranes even though hydrolysis of membrane protein by trypsin is restricted to the outer surface of the membrane (25–31); hydrolysis of superficial protein could perturb deeper layers of membrane structure. However, when trypsin-treatment was carried out in presence of Triton X-100 to make deeper layers of the membrane more readily accessible to trypsin, inactivation of the transferase proceeded in much the same manner as in the absence of detergent (Fig. 6). The overall extent of inactivation was only slightly greater in presence of Triton and the rate of inactivation was somewhat lower. When taken in conjunction with previous observations (13, 14, 19–21) of the freedom from latency of UDP-glucuronyltransferase in guinea pig microsomal membranes prepared with KCl, which are confirmed in the experiment of Fig. 6, these results support the view that the enzyme molecules are situated at or near the outer surface of these membranes.

The response of UDP-glucuronyltransferase of rat microsomal fractions prepared in KCl to trypsin was quite different from that of the guinea pig enzyme (Fig. 5). In the early stages of proteolysis some inactivation occurred but this was slower and less extensive than that observed with the guinea pig enzyme. Similar results were obtained using 50 and 100 μg of trypsin/mg of microsomal protein. Thus when the transferase was assayed with 0.6 mM *p*-nitrophenol and 4 mM UDP-glucuronate treatment of guinea pig membranes with protease lead to activities 70% less than those of controls in 10 min; the same treatment of rat membranes gave only 40% inactivation after 30 min and a similar difference was noted when the enzyme was assayed at the lower substrate concentrations. The difference in the magnitudes of inactivation is even greater than suggested by the above figures since the enzyme's activity in guinea pig microsomal fractions (30 nmol per min/mg of microsomal protein) is much higher than in rat fractions (6 nmol per min/mg of microsomal protein). Previous work (6–9, 11, 13, 14, 16) has shown that the enzyme of these rat microsomal preparations is highly latent, unlike that of the corresponding guinea pig membranes. This was confirmed by the present study; Triton X-100 activated the transferase in these preparations up to the values recorded with the guinea pig enzyme (Fig. 6). Detergent activation also markedly altered the response of the enzyme to trypsin treatment; in presence of Triton proteolysis caused inactivation of the transferase in a

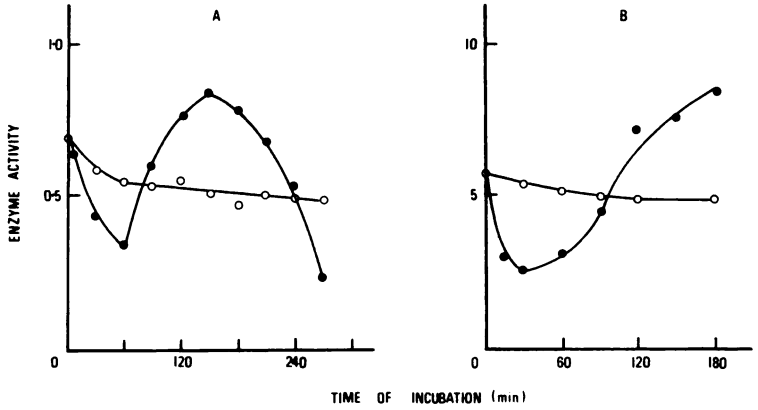

FIG. 5. *Effect of trypsin treatment on the UDP-glucuronyltransferase activity of rat microsomal membranes*
Microsomal suspensions were incubated with (●) and without (○) trypsin. (A) Portions of the digest were withdrawn after various times and assayed for transferase activity, at low substrate concentrations, in presence of trypsin inhibitor. (B) Portions of the digest were withdrawn and trypsin inhibitor added. Portions of these mixtures were then assayed for transferase activity at high substrate concentrations.

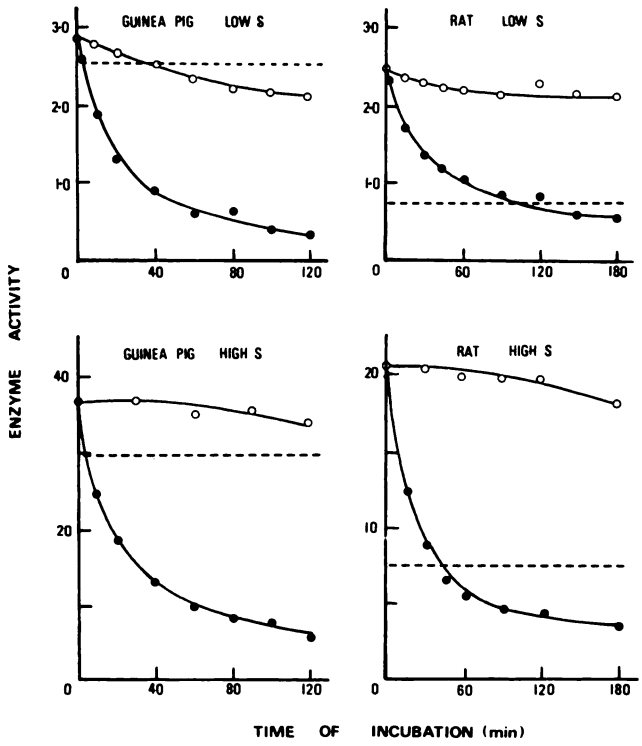

FIG. 6. *Effect of trypsin treatment in presence of 0.1% Triton X-100 on the UDP-glucuronyltransferase activities of rat and guinea pig microsomal membranes*

Microsomal suspensions were incubated with (●) and without (○) trypsin. When transferase assays were performed at low substrate concentrations, portions of the digests were withdrawn after various times and assayed in presence of trypsin inhibitor. With high substrate concentration assays, trypsin inhibitor was added to portions of the digests at the times indicated. Portions of these mixtures were then assayed for transferase activity. The dashed line indicates the transferase activity of untreated microsomal membranes.

fashion similar to that observed with guinea pig preparations. Hänninen and Puukka (8), also studying UDP-glucuronyltransferase of rat microsomal fractions isolated in 0.15 M KCl, reported a similar inactivation by trypsin after using digitonin to disrupt membrane structure and recent work (38) has demonstrated the same effect on rat and guinea pig enzymes in sucrose-prepared microsomal fractions when latency was removed by Triton X-100 or ultrasonication.

These data suggest that the UDP-glucuronyltransferase of guinea pig and rat liver microsomal membranes, prepared and washed with 154 mM KCl, are distributed differently within the membrane structures. The majority of enzyme molecules in guinea pig membranes appear to be situated at or near the external surface while these molecules in rat membranes seem to be located primarily nearer the luminal surface. However, the data could be explained by two other theories.

(1) It could be argued that on disruption of the liver endoplasmic reticulum during preparation of the microsomal membranes the vesicles might reseal with either their external surface ("right side-out") or their luminal surface ("inside-out") exposed to the bulk aqueous environment, leading to a mixed population of vesicles heterogeneous with respect to surface components. If the transferase were rigidly located at or near the luminal surface of the reticulum membrane our data would then suggest that the guinea pig preparation contained mainly "inside-out" membranes in which the transferase was exposed to trypsin, while the membranes prepared from rats were predominantly "right side-out" so that most of the transferase was protected by a membrane permeability barrier. If this the-

ory were correct, all microsomal enzymes located at or near the luminal surface of the endoplasmic reticulum would show the same difference in latency between guinea pig and rat preparations that we have observed for UDP-glucuronyltransferase. One such enzyme, nucleoside diphosphatase (28, 39) is, however, equally latent in both preparations as judged by its strong activation by detergents (Table 1). Indeed it is as latent in both these membrane preparations as it was in those used by Kuriyama (28) and Hallinan (39). (It is also noteworthy that the results in Table 1 confirm our previous observations [13, 14; see also Fig. 6] that in these preparations rat transferase is latent while guinea pig enzyme is almost fully active.) Thus, the differences we have observed between the transferase activities of guinea pig and rat preparations (both in latency and response to trypsin) cannot be due to differences in "sidedness" of the microsomal vesicles.

(2) A further theory proposes that the tranferase molecules are similarly located on the luminal surface of the microsomal vesicles in preparations from both guinea pig and rat, but that while the membrane in rat preparations is relatively impermeable to transferase substrates (particularly UDP-glucuronate) and to trypsin, the membrane in guinea pig preparations is permeable to these substances. As a result, the guinea pig transferase would show less latency than the rat enzyme and would be more susceptible to trypsin. However, the observation that the degree of latency of nucleoside diphosphatase is similar in guinea pig and rat (Table 1) indicates that the membrane in guinea pig preparations is no more permeable to UDP than in rat preparations. UDP is structurally similar to UDP-glucuronate, and moreover, the degree of latency of the reverse transferase reaction (UDP substrate) is similar to that of the forward reaction (UDP-glucuronate substrate) in all the microsomal fractions examined (Table 1). It is concluded that the guinea pig membrane is probably no more permeable to UDP-glucuronate than is the rat membrane. It is even less likely that there is a difference in permeability to trypsin, and it is most improbable that this "leaky membrane" theory can account for our observations.

Our general conclusion on UDP-glucuronyltransferase in rat microsomal fractions prepared in 154 mM KCl is that in intact vesicles most of the enzyme molecules are located so as not to be readily

TABLE 1

Latency of UDP-glucuronyltransferase and nucleoside diphosphatase in guinea pig and rat microsomal membranes

Latency was removed by treating the microsomal membranes with detergents (see the MATERIALS AND METHODS section). When latency of the transferase forward reaction and nucleoside diphosphatase was investigated, portions of the microsomal fractions were also treated with detergents in presence of 5 mM EDTA (an inhibitor of nucleoside diphosphatase (39)); transferase activities were close to those found in the absence of EDTA, while nucleoside diphosphatase activities were reduced by at least 70%. Latency of the transferase reverse reaction was investigated in separate experiments. The values in parentheses show the extents of detergent activation.

Microsomal fraction from	Detergent	UDP-glucuronyl-transferase activity	Nucleoside diphos-phatase activity	UDP-glucuronyl-transferase (reverse reaction) activity
		(nmol per min/ mg of protein)	*(nmol per min/ mg of protein)*	*(nmol per min/ mg of protein)*
Guinea pig	none	29.1	6.6	13.3
	Triton X-100	40.5 (1.39)	16.6 (2.52)	13.9 (1.05)
	Deoxycholate	50.7 (1.74)	13.4 (2.03)	— —
	Digitonin	— —	— —	15.2 (1.14)
Rat	none	11.5	11.0	4.2
	Triton X-100	41.9 (3.65)	31.1 (2.83)	7.6 (1.81)
	Deoxycholate	40.4 (3.52)	26.0 (2.57)	— —
	Digitonin	— —	— —	8.8 (2.10)

accessible to substrates, and so that they (and/or other membrane proteins which might affect transferase activity) are not accessible to trypsin. Triton X-100 has disrupted the microsomal membrane, increasing the availability of substrates to the transferase molecules, and exposing to the protease areas of the membrane that had been shielded including the transferase molecules themselves and any membrane proteins which might help maintain full transferase activity. Reactivation of rat transferase by phosphatidylcholine after trypsin inactivation was not attempted since prolonged modification of these microsomal preparations with phospholipase A is known to generate a thermolabile form of the enzyme (12) which is relatively insensitive to added phospholipid.[1]

Figure 5, which is typical of a number of experiments investigating the effects of trypsin on the UDP-glucuronyltransferase of rat microsomal preparations, shows that under these experimental conditions trypsin caused a triphasic effect. The initial slight inactivation was followed by an activation which in turn was followed by another, more pronounced, inactivation. This behavior of the enzyme has not been reported previously. Nilsson and Dallner (30, 31) observed that trypsin caused a slight inactivation of the transferase when sucrose-isolated, buffer-washed microsomal membranes were treated for up to 30 min. Wilkinson and Hallinan (38), on the other hand, reported a small stimulation of the enzyme's activity during 2 hr treatment of sucrose-isolated membranes with trypsin.

The initial inactivation of UDP-glucuronyltransferase in these intact rat microsomal membranes by trypsin, as discussed earlier, suggests that a small proportion of the enzyme molecules are situated at or near the outer surface where they are readily susceptible to trypsin. To account for the subsequent effects we propose the following scheme.

$$\begin{array}{ccc} A & \xrightarrow{k_1} & D \\ & & \uparrow k_3 \\ B & \xrightarrow{k_2} & C \end{array}$$

A represents the small proportion of transferase molecules which are exposed to the effects of trypsin and thus converted by proteolysis into a form D of low specific activity. B represents the majority of the transferase molecules which are deeply buried in the membrane and therefore not immediately susceptible to trypsin inactivation. In addition to inactivating form A, trypsin increases the permeability of the microsomal membrane to substrates [also proposed in ref. 38] though it does this less efficiently than detergents. As a result, the apparent activity of form B increases and this is represented by conversion into form C, although it must be recognized that this does not necessarily imply a change in the enzyme molecules themselves. Form C is expected to have similar properties to form A. This activation is manifested as the upward turn of the curve in Fig. 5. Before form C can exhibit its full activity, a further process supervenes, i.e., inactivation of form C by trypsin similar to the inactivation of form A. This is manifested in the final downward turn of the curve in Fig. 5a.

In all the experiments the rate of fall of transferase activity was first-order with respect to transferase activity. With guinea pig preparations in the absence of Triton X-100 the rate constant was abut 0.07 min^{-1}. With rat or guinea pig preparations in the presence of Triton X-100 it was about 0.04 min^{-1}. For the purpose of illustrative calculation we assume that the rate constant [k_1] for the conversion of form A into D in intact rat microsomal membranes has the value 0.08 min^{-1}. If we also assume that the conversions of form B into C and of form C into D also follow first order kinetics, the concentrations of these transferase forms during trypsin treatment can be expressed by the following equations (40),

$$[A] = [A]_0 e^{-k_1 t}$$

$$[B] = [B]_0 e^{-k_2 t}$$

$$[C] = [B]_0 \frac{k_2}{k_3 - k_2} (e^{-k_2 t} - e^{-k_3 t})$$

$$[D] = [B]_0 \left[1 - \frac{1}{k_3 - k_2} (k_3 e^{-k_2 t} - k_2 e^{-k_3 t}) \right] + [A]_0 (1 - e^{-k_1 t})$$

where the subscript zero indicates concen-

[1] A. B. Graham, D. T. Pechey and G. C. Wood, unpublished work.

tration at zero time of treatment. UDP-glucuronyltransferase activity (a) is given by

$$a = \alpha\{[A] + [C]\} + \beta[B] + \delta[D]$$

where α, β and δ are the specific activities of the different enzyme forms. To illustrate the form of the activity/time curve these equations predict, the treatment is simplified by assuming that forms B and D are inactive (i.e., $\beta = \delta = 0$) and that $\frac{[B]_0}{[A]_0} = 15$. The curve generated when k_1, k_2 and k_3 are 0.08, 0.002 and 0.018 min^{-1} respectively is shown in Fig. 7 and is very similar to that found experimentally (Fig. 5a). This finding is consistent with the hypothesis that, in rat microsomal fractions prepared in KCl, the majority of transferase molecules occupy sites deep within the membranes. However, further work is required to test fully this simple kinetic model.

The results presented in this paper provide strong evidence to suggest that differences between the UDP-glucuronyltransferases of guinea pig and rat liver microsomal membranes, prepared and washed in 154 mM KCl, in response to membrane perturbants can be explained by a difference in the intramembrane distribution of enzyme molecules.

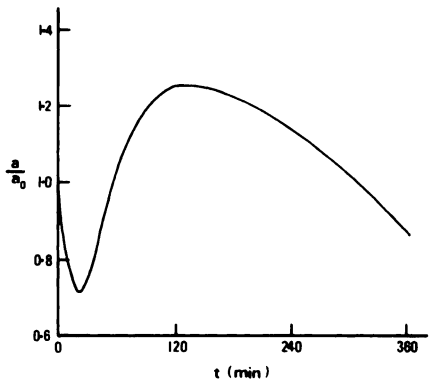

FIG. 7. *Effect of trypsin treatment on rat microsomal UDP-glucuronyltransferase activity predicted by a simple kinetic model*

Transferase activity during trypsin treatment was calculated using the equations and assumptions detailed in the text.

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