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KINETIC STUDIES OF LATENT MICROSOMAL UDP-GLUCURONYLTRANSFERASES

KINETICS OF GLUCURONIDATION IN INTACT AND PERTURBANT-TREATED MEMBRANES

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Double-reciprocal plots (with UDPglucuronate as varied substrate) of the rate of glucuronidation of *p*-nitrophenol by the latent UDPglucuronyltransferases of intact guinea pig and rat liver microsomal membranes (prepared with 154 mM KCl and 0.25 M sucrose) were continuously curved concave-downwards. Good fits to the kinetic data were obtained by using two different calculation methods which assume that two forms (high K and low K) of the transferase catalyse the reaction simultaneously. No evidence of cooperativity in binding of UDPglucuronate to the enzyme was found. When latency of the enzymes of these preparations was destroyed by disrupting the membranes with Triton X-100 or lysophosphatidylcholine, double-reciprocal plots were linear. With guinea pig membranes, lysophosphatidylcholine generated an activated single-enzyme form obeying the simple Michaelis-Menten rate law; K for the activated species was close to that (K_1) for the native low K form and its value of V was greater than the combined maximum velocities ($V_1 + V_2$) of the two forms in intact membranes. With rat membranes, both perturbants produced a single activated form also with $V > (V_1 + V_2)$ and with $K_2 > K > K_1$. These results are discussed and are consistent with the view of transferase latency which envisages that there are two populations (buried and exposed) of enzyme molecules in intact microsomal membranes. The effects of membrane perturbants on the kinetic parameters of the two native transferase forms were assessed by accounting for the possibility that the reactivity of the buried transferase is controlled by the rate of transport of UDPglucuronate across the membrane matrix. The data are compatible with a model which supposes that UDPglucuronate gains access to the buried population by a process with the kinetic characteristics of a facilitated transport system.

Introduction

UDPglucuronyltransferase (EC 2.4.1.17) is an integral enzyme, or group of related enzymes, of the endoplasmic reticulum membrane of liver cells catalysing the reversible transfer of the glucuronate grouping from UDPglucuronate (UDP-GlcUA) to a wide variety of poorly water-soluble, nucleophilic acceptors. Catalytic function is regulated by the enzyme's interaction with its membrane phospholipid environment.

Intact membrane phospholipid structure exerts two types of regulating influence on the enzyme, and these are superimposed upon one another [1–3]. Firstly, the transferase depends for full activity on its association with phospholipids. The requirements of purified transferases for phospholipids have been demonstrated recently [4–6] and it appears that phospholipids are essential for maintaining the enzyme in a reactive conformation [7]. However, in most microsomal preparations the enzyme is latent and intact phospholipid structure

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Abbreviations: UDP-GlcUA, UDPglucuronate; lysoPC, lysophosphatidylcholine.

also restricts activity. Membrane-perturbing agents have striking effects on microsomal transferase. Mild degrees of perturbation activate the enzyme by removing latency, while more severe treatments inactivate by disrupting interactions between the enzyme and essential phospholipids [1,2,8,9].

The mechanism of latency never has been established unequivocally, and two radically different theories have been evolved and debated [10–15]. One theory, that of 'compartmentation' (summarised in Ref. 12), is based on an extensive and diverse collection of evidence (see, for example, Ref. 13); it states that in intact microsomal membranes transferase molecules (or their active sites) are located in positions near the inside surface of the microsomal vesicle. Restriction of activity is considered to be due to the phospholipid bilayer substantially hindering access of substrates to the sites of catalysis. Membrane perturbants are envisaged to remove latency by destruction of the membrane permeability barrier.

The alternative theory of latency invokes an allosteric model where membrane phospholipids, perturbants and physiological activators (UDP-*N*-acetylglucosamine and bivalent metal ions) affect the conformation of transferase molecules located near the outer surface of microsomal membranes (summarised in Ref. 10). This 'conformational' concept was formulated almost entirely from kinetic studies of the membrane-bound transferase. Vessey and Zakim [16] observed that various perturbants produce highly active forms of the enzyme apparently with different kinetic properties and it was proposed that latency is the result of membrane phospholipids constraining the enzyme in a low-activity conformation; perturbants activate by releasing the enzyme from constraint with each perturbant causing the transferase molecules to adopt a different conformation. Latent transferase was shown to exhibit anomalous kinetics when UDP-GlcUA was the substrate varied, and Vessey et al. [17] reported that double-reciprocal plots deviate from linearity at low concentrations of UDP-GlcUA. Zakim et al. [18] ascribed this behaviour to conformational changes in enzyme structure resulting in cooperative kinetics. Further kinetic data from Zakim and his colleagues [18] led to the suggestion that UDP-*N*-acetylglucosamine increases the enzyme's affinity for UDP-GlcUA and that the activator is a positive allosteric effector [10,11].

These theories are fundamentally different and in previous work from this laboratory we have tried to distinguish between them and to resolve the mechanism of latency [8,9,19–23]. Evidence from the responses of latent and activated microsomal enzyme forms to membrane impermeant proteinases and to variation of temperature has been adduced in support of a slightly more elaborate variation of the 'compartmentational' model. We proposed that in microsomal membranes where the enzyme is latent only a small proportion of transferase molecules is located near the membrane outer surface, while the vast majority occupy deeply buried locations which become accessible to proteinases and substrates only after membrane perturbation [21,23]. Moreover, the degree of transferase latency appears to be related to the fraction of buried enzyme molecules [23].

The coexistence of two transferase forms (buried and exposed) within the membranes would generate kinetics which do not obey the simple Michaelis-Menten rate law. The double-reciprocal plots predicted would be curved [24,25] and would resemble those reported by Vessey et al. [17]. These workers interpreted this anomalous behaviour as negatively cooperative allosterism and rejected the idea that two enzyme forms might contribute simultaneously to the rates of glucuronidation they observed [18].

In this paper we reexamine closely the kinetics of latent microsomal UDPglucuronyltransferase by measuring rates of glucuronidation with a large number of different concentrations of UDP-GlcUA at a fixed concentration of acceptor (*p*-nitrophenol) to investigate the possibility that two transferase forms act simultaneously. Also by studying the kinetics of glucuronidation in membranes treated with perturbants, where any compartmentation is destroyed, rates should be predicted by a simple single-enzyme model if our variation of the 'compartmentation' theory is valid.

Materials and Methods

Chemicals. UDPglucuronate (triammonium salt), bovine serum albumin (crystalline), lysophosphatidylcholine (lysoPC, egg-yolk) and

Triton X-100 were from Sigma London Chemical Co., Poole, Dorset, U.K. All other chemicals were of analytical reagent grade if available.

Animals. Male rats (Wistar, 200–300 g) were from Animal Supply Ltd., Welwyn, Herts., U.K. Male guinea pigs (Hartley, 250–400 g) were from David Hall, Newchurch, Burton-on-Trent, Staffs., U.K.

Preparation of microsomal membranes. Animals (starved overnight) were killed and liver microsomal membranes were prepared and washed with 154 mM KCl [19] or 0.25 M sucrose [26]. The membranes were resuspended in small volumes of 154 mM KCl or 0.25 M sucrose and adjusted with these solutions to suitable concentrations (20–40 mg protein/ml for membranes in KCl, 20–70 mg/ml for membranes in sucrose). The protein contents of the membrane suspensions were determined with a biuret reagent [27] standardised with dried bovine serum albumin. The suspensions were stored at 0°C for no more than 18 h before being used.

Determination of UDPglucuronyltransferase activities. Initial rates of glucuronidation at 37°C were determined as described previously [19] with modifications [20]. Rates of reaction of a fixed concentration of *p*-nitrophenol (0.6 mM) were measured at fourteen different concentrations of UDP-GlcUA (0.1–20 mM) with a fixed amount of microsomal protein (2–15 mg) in the reaction mixtures. The enzyme concentration was selected by determining the transferase activity at 37°C using 0.6 mM *p*-nitrophenol and 4 mM UDP-GlcUA as soon as the membranes were prepared. At six appropriate times (0.5 to 4 min intervals), 0.1 ml portions of the reaction mixtures were removed to determine the amount of acceptor glucuronidated. Velocities (nmol/min per mg of microsomal protein) were calculated from initial rates.

Since rat membranes contain significant nucleotide pyrophosphatase activity, EDTA (10 mM, final concentration) was added to inhibit the hydrolysis of UDP-GlcUA when reactions contained rat microsomal protein [8].

Treatment of membranes with perturbants. Microsomal membranes were treated for 10 min at 20°C with lysoPC (0.3 μmol/mg of protein) or Triton X-100 (0.1%) as described previously [9]. These conditions produce near maximum activation of latent transferases [9,20]. The reaction mixtures used to determine rates of glucuronidation with perturbant-treated membranes contained 0.075–0.563 mM lysoPC or 0.0125% Triton X-100.

Analyses of kinetic data. Data were plotted initially in double-reciprocal form, i.e. as $1/v_o$ against 1/[UDP-GlcUA] where v_o is the initial velocity observed.

When these plots were linear, the data were fitted by computer to Eqn. 1 using an unweighted least-squares procedure.

$$v_o = \frac{V s}{K + s} \tag{1}$$

However, may sets of data yielded plots which were curved concave downwards (see Fig. 1). If two enzymes, or two forms of the enzyme in membranes, contribute simultaneously to v_o, and if the rate of reaction of each enzyme form follows the Michaelis-Menten rate law, v_o is given by Equation 2 [24,25],

$$v_o = \frac{V_1 s}{K_1 + s} + \frac{V_2 s}{K_2 + s} \tag{2}$$

the subscripts referring to the separate enzyme forms. Inverting Eqn. 2 yields Eqn. 3,

$$\frac{1}{v_o} = \frac{1 + (K_1 + K_2)\frac{1}{s} + K_1 K_2 \frac{1}{s^2}}{(V_1 + V_2) + (V_1 K_2 + V_2 K_1)\frac{1}{s}} \tag{3}$$

and at high concentrations of the substrate varied (s), this relationship approaches asymptotically a linear approximation (Eqn. 4)

$$\frac{1}{v_o} = \frac{1}{(V_1 + V_2)} + \left[\frac{V_1 K_1 + V_2 K_2}{(V_1 + V_2)}\right]\frac{1}{s} \tag{4}$$

which defines the part of the double-reciprocal plot where the enzyme form with the higher value of K (high-K form) makes its greatest contribution to v_o. At very low concentrations of substrate, Eqn. 3 approaches a second linear approximation (Eqn. 5)

$$\frac{1}{v_o} = \frac{V_1 K_2^2 + V_2 K_1^2{}_2}{(V_1 K_2 + V_2 K_1)} + \left[\frac{K_1 K_2}{V_1 K_2 + V_2 K_1}\right]\frac{1}{s} \tag{5}$$

where the enzyme form with the lower value of K

(low-K form) predominates. To obtain crude estimates for the kinetic parameters of the two enzyme forms, tangents were drawn to the asymptotically linear portions of the plots and first approximations to K_1, K_2, V_1 and V_2 were calculated from the values of their slopes and intercepts (see Eqns. 4 and 5). Two procedures were used to obtain more precise values for the kinetic parameters of the two putative enzyme forms. (a) Least-squares fit. The data were fitted to Eqn. 2 by an unweighted least-squares procedure which requires the first approximations of the values of K_1, K_2, V_1 and V_2 obtained by tangential approximation. (b) Osmundsen [28] method. The data also were fitted to Eqn. 2 by using Osmundsen's weighted non-linear regression approach. The computer program used was a modification of that supplied to us by Dr. H. Osmundsen, University of Oslo.

The data were processed by the ICL 1904/S computer of the University of Strathclyde Computer Centre with programs compiled in Fortran IV. The programs provided the values of the kinetic constants, the velocities predicted by these values at the concentrations of UDP-GlcUA used in the experiments, and an indication of the error between the computed fit and the experimental velocities by evaluating the sum of the squares of their deviations.

Results and Discussion

Kinetics of UDPglucuronyltransterase in untreated membranes

Typical double-reciprocal plots for the transferase of native guinea-pig and rat microsomal membranes prepared with 154 mM KCl and 0.25 M sucrose are shown in Fig. 1. Clearly, the data describe continuous curves which are concave-downwards. The curves drawn in Fig. 1 are excellent fits and were constructed from the values of the kinetic parameters (Table I) obtained by fitting the data to a two-enzyme model (Eqn. 2) by two different procedures.

Although all of the plots (Fig. 1) were curved, their individual shapes varied; the degree of curvature appears to depend on the degree of transferase latency associated with the membrane preparation. With the transferase of KCl-prepared guinea pig membranes (Fig. 1a), which has the lowest degree of latency (Table III), plots curved gently over the entire range of UDP-GlcUA concentrations. Plots for the transferase of rat membranes prepared with sucrose (Fig. 1d), which shows a high degree of latency, curved very strongly, and the remaining two microsomal preparations, which have intermediate and almost equal degrees of latency, gave plots (Figs. 1b and 1c) which were similar in shape and of intermediate curvature.

Table I gives the kinetic parameters calculated for the two proposed transferase forms from the results of three separate experiments with each type of microsomal membrane preparation. In most cases, the two calculation methods used (see Materials and Methods) yielded values which are in good agreement, but with one set of data (Table IA, Expt. 3) they furnished values which obviously were very different. This is the result of the two methods employing entirely different data-fitting procedures. The least-squares method computes the best fit to all of the experimental points, whereas the Osmundsen method [28] evaluates the kinetic parameters which describe the best fits at the high- and low-concentration ranges of UDP-GlcUA (where the two enzyme forms make their greatest contributions to v_o). Thus, the error in values of v_o affects the parameters evaluated by each method in a different fashion. With no error in v_o the kinetic parameters provided by both methods are identical; this was verified using hypothetical velocities generated by two enzyme forms with known kinetic parameters (Table IE).

The values of the kinetic constants of transferase form 1 (K_1, V_1) always were much smaller than those of form 2 (K_2, V_2) regardless of which microsomal preparation was used (Table I). V_2 was about 10-times larger than V_1 with all the microsomal preparations. When values of K for the two forms were compared, a trend emerged which appears to be connected with the degree of enzyme latency associated with each membrane preparation. For the weakly latent transferase of guinea pig KCl-prepared membranes, the ratio K_2/K_1 was about 10; for the highly latent transferase of sucrose-prepared rat membranes this ratio was approx. 250, and for the other two preparations, with intermediate degrees of latency, the ratios

were near 50. The degree of curvature of the double-reciprocal plots (Fig. 1) is related to the degree of latency and this is determined by the relative values of K_1 and K_2. We have confirmed theoretically that the curvature of the kinetic plots observed when two enzymes act simultaneously is determined only by the values of K for the two species (see also Ref. 24). Table I shows also that the values of K_1, V_1 and V_2 are largest for the transferase forms of guinea pig KCl-prepared membranes and smallest for those of rat sucrose-prepared membranes. The two other microsomal fractions have enzyme forms with intermediate values of K_1, V_1 and V_2 and again their

TABLE I

KINETIC PARAMETERS OF UDP-GLUCURONYLTRANSFERASE FORMS IN INTACT MICROSOMAL MEMBRANES

(A) denotes experiments with guinea pig membranes prepared with 154 mM KCl, (B) experiments with guinea pig membranes prepared with 0.25 M sucrose, (C) experiments with rat membranes prepared with 154 mM KCl, (D) experiments with rat membranes prepared with 0.25 M sucrose and (E) shows the parameters evaluated from hypothetical data generated by two enzyme forms with known kinetic parameters (experiment 1: $K_1 = 0.005$, $V_1 = 0.5$, $K_2 = 5.0$, $V_2 = 5.0$; experiment 2: $K_1 = 1.0$, $V_1 = 5.0$, $K_2 = 5.0$, $V_2 = 25.0$). (a) signifies parameters calculated by the least-squares method, (b) by Osmundsen's method. Values of K are expressed as mM UDP-GlcUA, values of V as nmol/min per mg of microsomal protein. See the text and Fig. 1.

Type of membranes	Expt. No.	Calculation method	Kinetic parameter			
			K_1	V_1	K_2	V_2
A	1	a	0.28	0.92	4.69	31.85
		b	1.34	5.59	6.26	28.68
	2	a	0.30	2.12	4.73	24.09
		b	0.18	1.40	4.26	24.20
	3	a	1.11	10.54	7.67	15.73
		b	0.11	1.06	2.85	21.93
B	1	a	0.29	3.08	5.96	5.13
		b	0.41	3.99	18.60	6.89
	2	a	0.24	1.33	2.14	4.55
		b	0.08	0.56	1.57	5.15
	3	a	0.29	1.05	35.17	12.71
		b	0.30	1.05	33.51	12.24
C	1	a	0.12	0.61	3.94	3.88
		b	0.20	0.87	5.29	3.92
	2	a	0.16	0.58	8.77	10.50
		b	0.12	0.52	8.35	10.31
	3	a	0.05	0.64	8.28	5.15
		b	0.08	0.75	10.28	5.50
D	1	a	< 0.01	0.28	8.60	3.23
		b	0.01	0.32	10.27	3.46
	2	a	< 0.01	0.20	4.85	2.92
		b	0.05	0.21	4.79	2.90
	3	a	< 0.01	0.10	2.02	1.87
		b	0.08	0.26	4.86	2.62
E	1	a	0.005	0.50	5.00	5.00
		b	0.005	0.50	5.00	5.00
	2	a	1.00	4.97	4.99	25.00
		b	0.84	3.65	4.60	26.00

magnitudes seem to be related to the degree of transferase latency; the less latent the enzyme, the greater the values of K_1, V_1 and V_2. Values of K_2 for the transferases of all four native membrane preparations were similar. These results support strongly our proposition that in native microsomal membranes two forms of the transferase (high-K and low-K forms) might catalyse simultaneously the glucuronidation of *p*-nitrophenol. Moreover, they show that the degree of enzyme latency observed in a microsomal preparation is reflected in the relative values of the kinetic parameters of the separate enzyme forms.

The anomalous kinetic behaviour of latent UDPglucuronyltransferases has been reported previously and various interpretations of these results have been discussed. Winsnes [29,30] published kinetic data which might have been best fitted by continuous concave-down curves. They had been obtained using a fixed concentration of *p*-nitrophenol (0.5 mM), various concentrations of UDP-GlcUA (0.25–4 mM) and rat and guinea pig liver microsomal fractions prepared with 154 mM KCl and were interpreted as being biphasic with two linear poretions intersecting sharply near 0.8 mM UDP-GlcUA. The difference in the slopes of the two apparently linear regions was large with rat enzyme and much smaller with guinea pig enzyme (cf., data of Fig. 1). Winsnes suggested [30] that these results might indicate that the latent transferases in intact membranes are subject to negatively cooperative binding of UDP-GlcUA, and was attracted to this explanation because it had been shown that when soluble enzymes exhibited negative homotropism in substrate binding, double-reciprocal plots were curves concave-down and the corresponding Hill plots ($\log(v_o/V - v_o)$ against $\log s$) had slopes (n) smaller than 1 [31].

In their studies of the latent microsomal transferase, Zakim and co-workers [17] published double-reciprocal plots of rate measurements made with native guinea pig membranes prepared with 0.25 M sucrose where the concentrations of acceptors (0.2 mM *p*-nitrophenol or 0.2 mM *o*-aminobenzoate) were fixed and those of UDP-GlcUA were varied. They recognised that the kinetics were unusual and they claimed that the plots were curvilinear, i.e. linear at UDP-GlcUA concentrations greater than 2.5 mM and curved concave-down below this value. These workers considered the possibility that two transferase isoenzymes might catalyse the reaction simultaneously, but ejected this notion on two major grounds [17]. Firstly, since their plots seemed to be linear from 2.5 to 40 mM UDP-GlcUA, they concluded that there was a single enzyme form only. It was argued that if there were more than one enzyme form, at higher concentrations of UDP-GlcUA the other form(s) of the transferase would have to be inhibited completely for linearity in this region to be observed, and that this was extremely unlikely. Secondly, addition of 2 mM UDP-*N*-acetylglucosamine had no effect on the intercept on the $1/v_o$-axis of the plots obtained by extrapolating to infinite substrate concentration the apparently linear region. However, the activator lowered the intercept formed by extrapolating the concave portion of the plots and it was stated that for two enzyme forms to account for this, UDP-*N*-acetylglucosamine would have had to increase the activity of the low-K isoenzyme at V. Thus it also would have had to inhibit the activity at V of any high-K form equally so as not to alter the intercept at the lower values of $1/v_o$. It was concluded finally that to explain their results using a two-enzyme model "too many qualifying assumptions would have to be made". However, on close inspection, the data of Vessey et al. [17] are better described by continuously curved double-reciprocal plots. Also, when it is realised that this graphical method 'clusters' data points towards the high concentration end of the plot and enhances the appearance of linearity in this region (see Fig. 1) it is reasonable to conclude that these plots are not curvilinear. Moreover, when two enzyme forms act simultaneously, double-reciprocal plots are continuously curved and the individual kinetic constants can not be obtained by extrapolating the regions tending towards linearity i.e. at high or low concentrations of the varied substrate [24,25]; the value claimed to be $1/V$ for the low-K species (intercept on the $1/v_o$-axis) is, in fact, $(V_1K_2^2 + V_2K_1^2)/(V_1K_2 + V_2K_1)^2$ (see Eqn. 5). Thus, Zakim's group rejected the two-enzyme model without fully understanding the kinetics it predicts.

Zakim and colleagues [17] proceeded in their

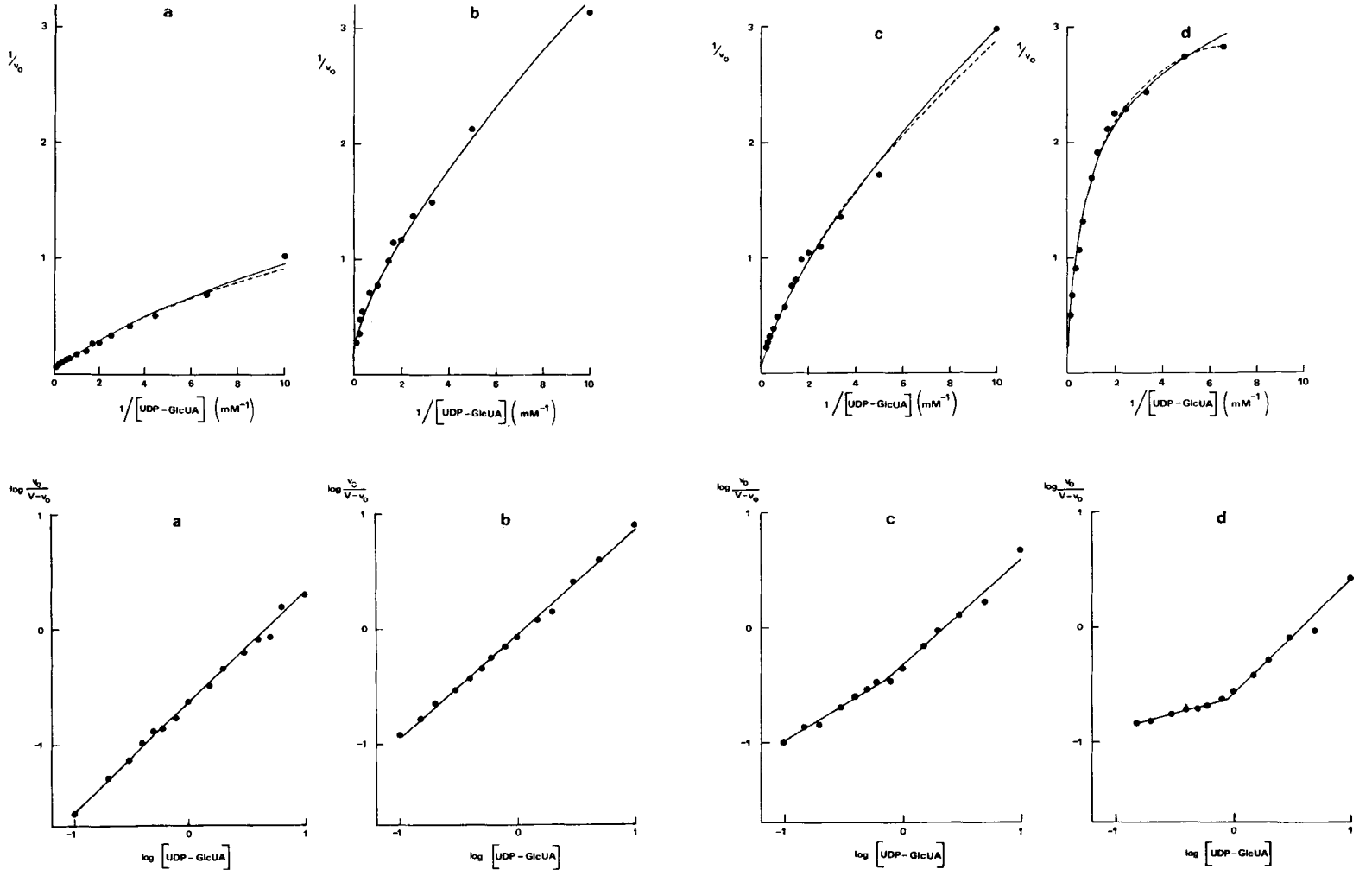

Fig. 1. (Upper row) Double-reciprocal plots of the rate of glucuronidation of *p*-nitrophenol in intact microsomal membranes with UDP-GlcUA as varied substrate. Initial rates with a fixed concentration of acceptor (0.6 mM *p*-nitrophenol) were determined as described in Materials and Methods; v_0 is expressed as nmol of acceptor glucuronidated per min per mg of microsomal protein. The dashed line gives the best fit by Osmundsen's method, the solid line the best least-squares fit. (a) Guinea pig membranes prepared with 154 mM KCl; (b) guinea pig membranes prepared with 0.25 M sucrose; (c) rat membranes prepared with 154 mM KCl; (d) rat membranes prepared with 0.25 M sucrose.

Fig. 2. (Lower row) Hill plots of the rate of glucuronidation of *p*-nitrophenol in intact microsomal membranes with UDP-GlcUA as varied substrate. See the legend of Fig. 1. V(nmol/min per mg of microsomal protein) is the initial rate of reaction at infinite [UDP-GlcUA] (i.e. $V_1 + V_2$, see Eqn. 4).

discussions of these anomalous kinetics by proposing that their interpretation of the data could be explained without recourse to 'additional qualifying assumptions' if it was assumed that the transferase is a multisubunit protein displaying negative cooperativity in the binding of UDP-GlcUA. Subsequently, they presented two Hill plots of their results so as to determine the extent of cooperative interaction between the substrate and their postulated single-enzyme entity [18]. These representations were stated to be linearly biphasic with limiting slopes (Hill coefficients, n) of 1 at higher concentrations of UDP-GlcUA, and below 1 at lower concentrations. It was concluded that this evidence confirmed negative cooperativity. Nevertheless, if these Hill plots are examined carefully, it is not unreasonable to suggest that the plot with *p*-nitrophenol as acceptor is linear ($n \simeq 1$) over almost the entire range of UDP-GlcUA concentrations. These authors did not offer an explanation for the clearly biphasic plot with *o*-aminobenzoate; this phenomenon cat not be explained by negative cooperativity alone.

Our kinetic data are more extensive and have been analysed more thoroughly than those reported previously for latent microsomal UDPglucuronyltransferases. They are compatible with a two-enzyme model. Nevertheless, the curved double-reciprocal plots of Fig. 1 might be the consequence of cooperativity, even although this is questionable in the case of a membrane-bound enzyme [32]. To try to distinguish between these models we also have constructed Hill plots of our data. Typical plots are shown in Fig. 2 and the Hill coefficients from replicate experiments are in Table II. Hill plots for the guinea pig transferase were linear from 0.1 to 10 mM UDP-GlcUA with slopes (n) of 0.93 ± 0.04 (S.D.) for KCl-prepared membranes and 0.86 ± 0.03 for sucrose-prepared membranes. If an enzyme exhibits negative cooperativity its Hill plot should be linear over a wide range of substrate concentrations with n below 1. Indeed, to be certain of this it is considered that n should be below 0.8 [33]. Thus, for the native guinea pig transferase there is no evidence from Hill plots of negative cooperativity. In the case of rat membranes, Hill plots were biphasic with slopes of 0.47 ± 0.22 at low concentrations of UDP-GlcUA and 1.06 ± 0.06 at higher concentrations. Again, these can not be explained by negative cooperativity. A plausible explanation, however, is that two enzyme forms with vastly different values

TABLE II

HILL COEFFICIENTS (n) FOR THE GLUCURONIDATION OF *p*-NITROPHENOL BY UDP-GLUCURONYLTRANSFERASE OF INTACT MICROSOMAL MEMBRANES

See Fig. 2, Table I and the text.

Animal	Membrane preparation	Expt. No.	Value of n		
			Low [UDP-GlcUA]	High [UDP-GlcUA]	All [UDP-GlcUA]
Guinea pig	154 mM KCl	1	–	–	0.89
		2	–	–	0.96
		3	–	–	0.93
	0.25 M sucrose	1	–	–	0.88
		2	–	–	0.81
		3	–	–	0.88
Rat	154 mM KCl	1	0.74	1.04	–
		2	0.65	1.04	–
		3	0.41	1.02	–
	0.25 M sucrose	1	0.26	1.11	–
		2	0.19	1.15	–
		3	0.56	0.98	–

of K contribute simultaneously to v_o, and we have confirmed that this circumstance generates data which lead to steeply-curved double-reciprocal plots which can not be linearised by logarithmic plots (cf. Figs. 1c and 1d with Figs. 2c and 2d; see also the data of Ref. 34). These findings might lend further support for the two-enzyme model. It is crucial to understand that a two-enzyme model and negative cooperativity generate identical substrate-binding isotherms, and Hill plots alone can not be used to distinguish between them [33]. For the two-enzyme model Hill coefficients can vary from 1 when K_1 is similar to K_2 (e.g., with KCl-prepared guinea pig membranes, see Table I), to values below 1 when K_1 and K_2 are different (guinea pig sucrose-prepared membranes). For the latent rat enzyme a new case arises where K_1 and K_2 are vastly different, and this situation results in biphasic Hill plots.

Kinetics of UDPglucuronyltransferase in membranes treated with perturbants

Typical double-reciprocal plots for the activated UDPglucuronyltransferases of perturbant-treated microsomal membranes are shown in Figs. 3 and 4. Plots for the guinea pig enzyme in KCl-prepared membranes treated with Triton X-100 were slightly curved concave-down (Fig. 3a); the kinetic parameters of the transferase forms calculated by fitting the data to a two-enzyme model (Eqn. 2) are shown in Table III. When this microsomal preparation was treated with lysoPC the kinetic plot was linear (Fig. 3b). With all the other microsomal preparations (treated with lysoPC or Triton) the plots were linear also (Figs. 3c and 3d, Figs. 4a–4d) and this suggests that removal of latency by perturbants generates a single enzyme form(s) which obeys the Michaelis-Menten rate law (Eqn. 1). The value of K did not vary greatly in the

TABLE III

KINETIC PARAMETERS OF UDP-GLUCURONYLTRANSFERASE IN MICROSOMAL MEMBRANES TREATED WITH PERTURBANTS

When guinea pig membranes prepared with 154 mM KCl were treated with Triton X-100, double-reciprocal plots were curved (see Fig. 3a) and the kinetic parameters of the two enzyme forms were calculated using the least-squares method. For all the other microsomal preparations the plots were linear and K and V were evaluated as described in Materials and Methods. Values of K are expressed as mM UDP-GlcUA, values of V as nmol/min per mg of microsomal protein. Transferase activity was determined with 0.6 mM *p*-nitrophenol and 5 mM UDP-GlcUA before perturbation and the extent of activation (latency) was assessed as the percentage increase in activity at this concentration of donor substrate (see also the data of Ref. 20). See Fig. 3, Fig. 4 and the text.

Animal	Membrane preparation	Perturbant	Expt. No.	Activation (%)	Kinetic parameter					
					K	V	K_1	V_1	K_2	V_2
Guinea pig	154 mM KCl	Triton X-100	1	85	–	–	0.69	11.32	7.97	23.6
			2	65	–	–	0.09	3.88	2.68	25.5
		lysoPC	1	110	1.11	41.67	–	–	–	–
	0.25 M sucrose	Triton X-100	1	320	0.46	31.25	–	–	–	–
			2	400	0.32	27.03	–	–	–	–
		lysoPC	1	460	0.82	42.02	–	–	–	–
Rat	154 mM KCl	Triton X-100	1	260	1.25	10.00	–	–	–	–
			2	200	1.88	10.00	–	–	–	–
		lysoPC	1	350	2.21	17.86	–	–	–	–
	0.25 M sucrose	Triton X-100	1	550	1.78	7.69	–	–	–	–
			2	600	1.47	6.67	–	–	–	–
		lysoPC	1	760	1.96	13.05	–	–	–	–

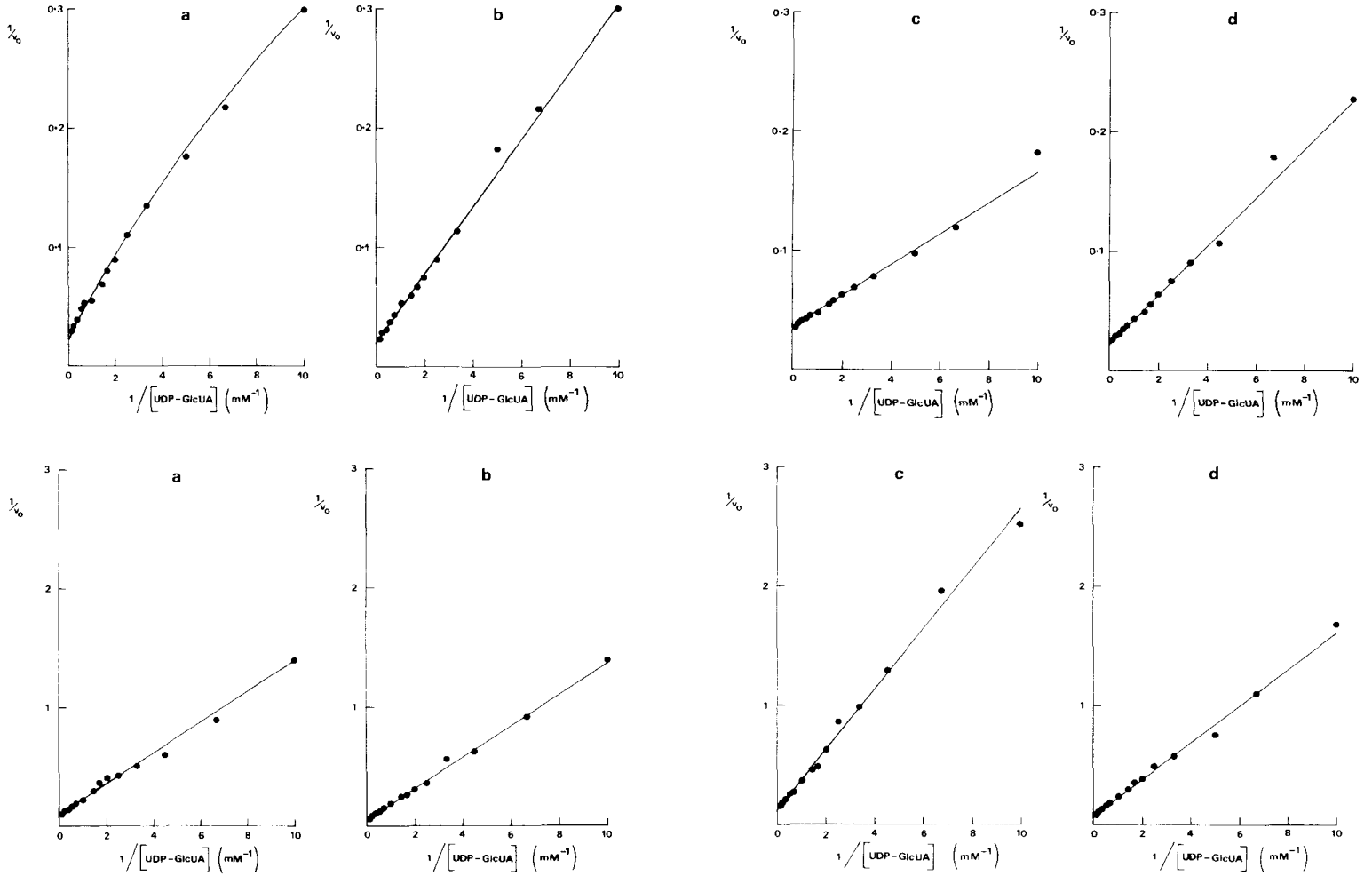

Fig. 3. (Upper low) Double-reciprocal plots of the rate of glucuronidation of *p*-nitrophenol with UDP-GlcUA as varied substrate in guinea pig microsomal membranes treated with perturbants. Initial rates with a fixed concentration of *p*-nitrophenol (0.6 mM) were determined as described in Materials and Methods; v_0 is expressed as nmol of acceptor glucuronidated per min per mg of microsomal protein. (a) Membranes prepared with 154 mM KCl treated with 0.1% Triton X-100; (b) membranes prepared with 154 mM KCl treated with lysoPC (0.3 μmol/mg of protein); (c) membranes prepared with 0.25 M sucrose treated with 0.1% Triton X-100; (d) membranes prepared with 0.25 M sucrose treated with lysoPC (0.3 μmol/mg of protein). Plot (a) is curved and the fit shown was calculated using the two-enzyme least-squares method (see Materials and Methods). The remaining plots are linear and the fits were calculated as described in Materials and Methods.

Fig. 4. (Lower row) Double-reciprocal plots of the rate of glucuronidation of *p*-nitrophenol with UDP-GlcUA as varied substrate in rat microsomal membranes treated with perturbants. See the legend of Fig. 3. All the plots are linear and the fits shown were calculated as described in Materials and Methods.

various perturbant-activated preparations (Table III); its full range for all the activated single forms was 0.32–2.21 mM. V for the single forms was similar in both types of guinea pig membrane. Similar values of V were observed with both types of rat membrane and this value was lower than that for the guinea pig enzyme.

When the kinetic characteristics of the latent native enzyme forms (Table I) are compared with those of the single-activated forms (Table III), it is seen that Triton and little or no effect on the two transferase forms of guinea pig KCl-prepared membranes while lysoPC removed latency and generated an enzyme form exhibiting Michaelis-Menten kinetics. K for the activated form (1.11 mM) was within the range of values of the low-K form in untreated membranes ($K_1 = 0.11–1.34$ mM) and V (41.67 nmol/min per mg of protein) was greater than the combined maximum velocities of the two native forms ($V_1 + V_2 = 28.04 \pm 4.48$ nmol/min per mg of protein). Both perturbants produced the same effects on the transferase of guinea pig sucrose-prepared membranes. The values of K for the activated form (0.32–0.82 mM) were close to the range observed for the native low-K form ($K_1 = 0.08–0.41$ mM). V for the Triton-activated form (27–31 nmol/min per mg of protein) and the lysoPC-activated form (42.02 nmol/min per mg of protein) were much larger than ($V_1 + V_2$) for the native forms (9.76 ± 3.51 nmol/min per mg of protein). With the transferase of rat membranes (prepared with KCl or sucrose) the situation was somewhat different. LysoPC and Triton had similar effects, but K for the activated forms resembled neither K_1 nor K_2; it had an intermediate value. K ranged from 1.25 to 2.21 mM compared with $< 0.01–0.20$ mM for K_1 and 3.94–10.28 mM for K_2. V for the Triton-activated form (10 nmol/min per mg of protein) and the lysoPC-activated form (17.86 nmol/min per mg of protein) of KCl-prepared membranes both were greater than ($V_1 + V_2$) [7.21 ± 2.98 nmol/min per mg of protein) and this was seen also for sucrose-prepared membranes. V after Triton-treatment was 6.67–7.69 nmol/mn per mg of protein, and ($V_1 + V_2$) was 3.28 ± 1.41 nmol/min per mg of protein. Thus, the two perturbants produced similar effects on the kinetic parameters of the two putative transferase forms of native membranes. Both generate apparently single highly active enzyme forms with similar values of K and values of V greater than ($V_1 + V_2$). Under these experimental conditions, however, lysoPC is a more efficient activator than Triton X-100.

Vessey and Zakim [26] studied the effects of Triton X-100 on the kinetics (at concentrations of UDP-GlcUA above 2 mM) of the latent transferase of guinea pig microsomal membranes prepared with sucrose. The activated enzyme form and the native transferase displayed Michaelis-Menten kinetics and, by assuming a simple one-enzyme model free from diffusion control, it was claimed that the enzyme's 'affinity' for UDP-GlcUA was increased selectively because the detergent apparently affected K (decreased 25-fold) without altering V. (At this time these authors were not yet aware that the latent transferase of intact membranes possessed anomalous kinetics (see Ref. 17)). When these data were analysed according to a bisubstrate kinetic mechanism [26] they found that Triton had reduced V slightly. Also it was claimed that when latency was removed with phospholipase A_2 a different activated form with different kinetic characteristics was generated. Phospholipase A_2 appeared to increase both the 'affinity' for UDP-GlcUA (i.e., decrease K) and V of the native enzyme, and in a later report [35] it was proposed that Triton and the phospholipase increased the 'affinity' for the substrate by different mechanisms. It was concluded also [35] that removal of latency with lysoPC affected the kinetics of the latent enzyme in a fashion resembling phospholipase A_2. From these results an elaborate theory [10] was formulated to explain latency of microsomal UDPglucuronyltransferase. This theory of 'conformational constraint' contends that membrane phospholipids constrain the enzyme in a low activity (latent) conformation and perturbants remove latency by affecting lipid-enzyme interactions and produce a variety of high-activity conformers each with individual kinetic and regulatory properties.

The proponents of the alternative theory of latency ('compartmentation', see the Introduction) have suggested that all perturbants should have the same effect on the latent enzyme and that this should be primarily to increase V for glucuronidation reactions by destroying compartmentation and

TABLE IV

HILL COEFFICIENTS (n) FOR THE GLUCURONIDATION OF p-NITROPHENOL BY UDP-GLUCURONYLTRANSFERASE OF MICROSOMAL MEMBRANES TREATED WITH PERTURBANTS

See Fig. 3, Fig. 4 and the text.

Animal	Membrane preparation	Perturbant	Expt. No.	Value of n at all [UDP-GlcUA]
Guinea pig	154 mM KCl	Triton X-100	1	1.00
			2	0.93
		lysoPC	1	1.09
	0.25 M sucrose	Triton X-100	1	0.88
			2	1.10
		lysoPC	1	0.97
Rat	154 mM KCl	Triton X-100	1	0.90
			2	0.93
		lysoPC	1	0.97
	0.25 M sucrose	Triton X-100	1	1.00
			2	1.06
		lysoPC	1	0.97

facilitating access to UDP-GlcUA to deeply buried intra-membrane catalytic sites [13,14].

The results described here (Figs. 3 and 4, Table III) show that removal of transferase latency in liver microsomal membranes form two animal species prepared by two methods with two different perturbants caused similar alterations in the kinetic parameters of glucuronidation at both K and V. These findings, clearly, do not support the concept of an allosteric enzyme with conformational flexibility responding selectively to different perturbants. This conclusion is reinforced by the observation (Table IV) that after treatment with perturbants the transferase exhibited linear Hill plots (not shown) with slopes close to 1 over a wide range of UDP-GlcUA concentrations. We have extended the 'compartmentation' theory to accommodate the idea that two forms of the transferase molecule (buried and exposed) coexist in intact microsomal membranes (Refs. 21,23; see also the Introduction). Thus, glucuronidation in native membranes would be catalysed by two enzyme forms acting simultaneously; the data presented here are entirely consistent with this view.

In order to interpret the effects of perturbants on the kinetic parameters of the two native transferase forms, the possibility that the activity of the buried transferase form is controlled by diffusion must be taken into account. Because the membrane phospholipid matrix is likely to restrict premeation of UDP-GlcUA to these catalytic sites, the rate of its transport must alter the reactivity of the buried form. For simple diffusion the partition coefficient (P) for UDP-GlcUA (the ratio of its concentration within the membrane to that in the surrounding bulk phase) and its rate of diffusion become important factors influencing the kinetics of this enzyme form [36,37]. The activities of soluble enzymes immobilised in gels or included in polymer matrices are affected by this form of control [37,38] and the kinetic equations which describe diffusion-limited catalysis also appear to be applicable to compartmentalised membrane enzymes [39,40]. The rate equation for such enzymes (Eqn. 6)

$$v_o = \frac{Vs}{K' + s} \qquad (6)$$

is similar to that of Michaelis and Menten but the term K' is no longer a parameter of the catalyst alone. V is not affected because sufficiently high concentrations of substrate can reach the buried

enzyme by simple diffusion; K' is a function of the true constant (K), P and the rate of substrate diffusion (Eqn. 7).

$$K' = \frac{K}{PF} \tag{7}$$

F is the dimensionless Thiele function which incorporates the diffusion coefficient for permeation of substrate (D) through a membrane of thickness l (Eqns. 8 and 9).

$$F = \frac{\tanh \partial l}{\partial l} \tag{8}$$

$$\partial = \frac{1}{2}\left(\frac{V}{DK}\right)^{\frac{1}{2}} \tag{9}$$

F has the property that when ∂l is small, F approaches 1 [37]. Thus, when l is small, K' becomes K/P. For microsomal membranes l must be small and K is liable to be modified by $P_{\text{UDP-GlcUA}}$. Since the sugar nucleotide is a fairly large, negatively charged, water-soluble molecule, P must be far below 1, and thus the value of K determined for the buried enzyme form (i.e., K_2, see below) must be larger than the true value.

It is now well-established that membrane perturbants disrupt microsomal membrane structure. Thus when latency of the native enzyme is destroyed, increased permeation of UDP-GlcUA would be expected to decrease the value of K observed for the buried form and to have very little effect on this parameter describing the exposed form. Moreover, if the two transferase forms in native membranes are identical, perturbants should produce a single enzyme form obeying the classical equation (Eqn. 1) with a value of K close to that of the exposed enzyme, if the only effect of perturbants is to destroy the permeability barrier to UDP-GlcUA. The data for the latent guinea pig enzyme demonstrate that the perturbants produced a single enzyme form with a value of K close to K_1. K_2 is decreased i.e., the high-K native form has vanished. It is reasonable, therefore, to conclude that in intact membranes (a) enzyme form 2 is buried, while form 1 is exposed, and (b) both native forms are identical, at least as far as K is concerned. However, it is important to note that perturbant treatments of guinea pig membranes generated a single enzyme form with $V > (V_1 + V_2)$. This would not be expected if the only effect of perturbants was to increase the rate of simple substrate diffusion to the buried enzyme form.

With rat membranes perturbants also produced a single activated form with $V > (V_1 + V_2)$. Its value of K was intermediate between K_1 and K_2. Here it is possible that the two native forms are not identical; although perturbants decreased K_2, the true value of K of form 2 might not be identical to K_1. If double-reciprocal plots generated by two enzyme forms are to be curved sufficiently to allow a confident estimation of the individual parameters of Eqn. 2, K_1 must differ from K_2 by a factor of at least 5[28]; almost linear plots are observed when the two forms have values of K which differ by less than this. The single value of K estimated by fitting such data to the Michaelis-Menten equation would yield a value intermediate between K_1 and K_2. For the rat enzyme it is possible that perturbants remove latency to reveal two catalytically different enzyme species. However, the possibility can not be ignored that perturbants might affect the activated enzyme form so as to increase K_1 to K.

Since simple diffusion control of the buried enzyme form can not explain the finding that $V > (V_1 + V_2)$, the effect of facilitated transport of UDP-GlcUA to the buried enzyme form must be considered. It is noteworthy that certain supporters of the 'compartmentation' theory have proposed that a permease system is involved in the transport of UDP-GlcUA across the membrane matrix [13,14,41]. A kinetic model for facilitated transport of a substrate through a membrane followed by transformation by a compartmentalised enzyme has been developed [42]. Here, the unidirectional flux of substrate is represented by a Michaelis-Menten type relationship (Eqn. 10)

$$J = \frac{V_T s}{K_T + s} \tag{10}$$

where J is the rate of flux, s is the concentration of substrate at the side of the membrane were flow originates, V_T is the maximum rate of flux across the membrane and K_T is the reciprocal of the affinity of the transporter for substrate. At steady-state the net flux of substrate is equal to the

rate of the enzyme-catalysed reaction. Providing the compartmentalised enzyme obeys the Michaelis-Menten rate law, its velocity can be represented by Eqn. 11

$$\frac{V_T s_e}{K_T + s_e} - \frac{V_T s_i}{K_T + s_i} = \frac{V_b s_i}{K_b + s_i} \quad (11)$$

where s_e and s_i are the external and internal substrate concentrations, K_b is the Michaelis constant of the buried enzyme and V_b is its maximum velocity. Engasser and Horvath [42] introduced also three dimentionless quantities (Eqns 12–14)

$$\beta = s/K_b \quad (12)$$

$$\nu = V_T/V_b \quad (13)$$

$$\chi = K_T/K_b \quad (14)$$

and transcribed Eqn. 11 into a dimensionless form (Eqn. 15)

$$(\chi + \beta_e + \nu\chi)\beta_i^2 + \chi(\chi + \nu + \beta_e - \nu\beta_e)\beta_i - \nu\chi\beta_e = 0 \quad (15)$$

where β is the dimensionless substrate concentration, ν is the activity modulus and χ is the affinity modulus. The model predicts that s_i (the concentration of substrate available to the buried enzyme) 'plateaus' at high values of s_e. Thus, the enzyme's activity 'plateaus' also, and usually $V_b > V_T$. When perturbants increase permeation of substrates they would be expected to render the transporter redundant; therefore, they would be expected to eliminate rate-limitation of the buried enzyme and, after membrane disruption, V for the single activated form should be greater than $(V_1 + V_2)$. Indeed this is the case for both rat and guinea pig enzymes. The maximum value of s_i decreases with increasing values of χ (i.e., with increasing affinity of the transporter for substrate) because the carrier would compete more successfully for the substrate within the membrane. The rate of reaction by the buried enzyme also depends on χ, and when $\chi \neq 1$, its activity is constrained. Small values of χ (i.e. small values of K_T) predict that the buried enzyme would be severely constrained. However, the data obtained with all four types of microsomal preparation show that V is not massively greater than $(V_1 + V_2)$, therefore we assume that $K_T > K_b$, but not greatly so. Thus, this model predicts that when latency of the transferase is destroyed by perturbants, the apparent value of K for the buried enzyme (K_2) should be decreased. Our results show quite clearly that K_2 is decreased in the direction of K_1. For guinea pig membranes, K is close to K_1, suggesting that the two native species are catalytically similar; for rat membranes K lies somewhere between K_1 and K_2 and it is possible that the two transferase forms are catalytically different. These results are consistent with our view that there are two populations of transferase molecules in intact microsomal membranes where the enzyme is latent. In addition, they are compatible with the proposal that UDP-GlcUA reaches the population of buried enzyme molecules by a process of facilitated transport.

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