The Phospholipid-Dependence of Uridine Diphosphate Glucuronyltransferase

TEMPERATURE-DEPENDENCE OF MICROSOMAL ENZYME ACTIVITY AND THERMOTROPIC CHANGES IN MEMBRANE STRUCTURE

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Arrhenius plots of the non-latent UDP-glucuronyltransferase (*p*-nitrophenol acceptor) activity of guinea-pig liver microsomal membranes prepared with 154-mM-KCl were linear from 5 to 40°C. Arrhenius plots for other microsomal preparations from guinea pig and rat liver that show various degrees of transferase latency, exhibited two linear regions intersecting at a sharp transition point near 20–25°C. This discontinuity was abolished or greatly decreased when transferase latency was removed by treating the membranes with perturbants of phospholipid bilayer structure. The fluorescent probe *N*-phenyl-1-naphthylamine detected a thermotropic change in the fluidity of the phospholipid acyl chains of all the microsomal membrane preparations studied, at temperatures close to those of the Arrhenius-plot transitions. It is concluded that the thermotropic change in the structure of the membrane bilayer probably is a 'phase separation' or clustering of phospholipids, which affects a permeability barrier that restricts access of substrate to the transferase molecules.

Liver microsomal UDP-glucuronyltransferase (EC 2.4.1.17) is modulated in two ways by the intact phospholipid structure of native microsomal membranes (Graham *et al.*, 1974a; Berry & Hallinan, 1976; Graham *et al.*, 1977). In certain microsomal preparations, especially those isolated with 0.25M-sucrose, phospholipids restrict activity, and recent evidence (see Graham *et al.*, 1977; Wilkinson & Hallinan, 1977) favours the view that transferase molecules are located deep within the microsomal membranes so that phospholipids act as part of a hydrophobic barrier hindering access of reactants to the sites of glucuronidation. Mild perturbation of phospholipid structure probably activates latent UDP-glucuronyltransferase by increasing membrane permeability (Berry *et al.*, 1976; Graham *et al.*, 1977; Wilkinson & Hallinan, 1977).

Transferase activity is much less latent when microsomal fractions are isolated and washed with 154-mM-KCl, and in guinea-pig microsomal membranes prepared by this method the enzyme is highly active, almost free from latency (Graham & Wood, 1972, 1973) and appears to be located near the outside surface of microsomal vesicles, where it is not greatly affected by permeability (Wood & Graham, 1972; A. B. Graham, D. T. Pechey & G. C. Wood, unpublished work). In this preparation UDP-glucuronyltransferase requires intact phospholipid for full activity (Graham *et al.*, 1977); degra-

dation of phospholipids or membrane delipidation inactivates this enzyme form and choline phospholipids restore high activity.

In microsomal fractions containing latent enzyme these dual modulating effects are superimposed, and phospholipid modification causes two opposing effects on the transferase: activation of latent enzyme by disruption of the permeability barrier and inactivation of enzyme released from latency by destruction of essential phospholipid (Berry *et al.*, 1976; Graham *et al.*, 1977).

It is reasonable to expect that transferase activity in membranes where the enzyme is restricted by permeability will have a different temperature-dependence from that in preparations where the barrier is absent or has been removed by membrane perturbants. Moreover, the phospholipid component of microsomal membranes undergoes some form of structural change over a narrow temperature range near 20–25°C (Lyons, 1972; Raison, 1973; Lee, 1975; Charnock & Bashford, 1975) and this might affect enzyme activity. Indeed many membrane-bound enzymes exhibit non-linear Arrhenius plots of activity, with sharp changes of slope or discontinuities that coincide with phospholipid structural transitions detected by spectroscopic techniques (Lyons, 1972; Raison, 1973; Sechi *et al.*, 1973; Lee, 1975; Charnock & Bashford, 1975; Bashford *et al.*, 1976; Melchior & Steim, 1976). Therefore we have

examined the temperature-dependence of enzyme activity in the different microsomal fractions to gain further insight into these modulation mechanisms.

Norling & Hänninen (1974) studied the temperature-dependence of enzyme activity in sucrose-prepared rat liver microsomal fractions, but only at high temperatures (above 26°C), where thermotropic alterations to the phospholipid bilayer are not observed. Eletr *et al.* (1973) determined enzyme activity of sucrose-prepared guinea-pig liver microsomal preparations over a wider temperature range. Arrhenius plots showed separate linear regions with a discontinuity near 19°C; the apparent activation energy (E_a) was higher above than below this temperature. They also incorporated lipophilic e.s.r. probes into the microsomal membranes and detected an abrupt increase in membrane fluidity as the temperature was raised above 19°C, and concluded that it was this thermotropic structural change that abruptly increased E_a . Later, however, Zakim & Vessey (1975) reported a discontinuity in Arrhenius plots at 16°C and their data indicated that E_a now was lower above the transition temperature than below it.

We present the results of a careful re-investigation of the temperature-dependence of UDP-glucuronyltransferase activity in rat and guinea-pig liver microsomal membranes prepared with both 0.25M-sucrose and 154mM-KCl. We also describe the temperature-dependence of latent enzyme after it has been activated with phospholipase A (EC 3.1.1.4), its phospholipid hydrolysis products or Triton X-100, and have attempted to relate these kinetic data to a thermotropic change in membrane organization observed by labelling microsomal membranes and aqueous dispersions of their phospholipids with two fluorescent probes of phospholipid structure (Radda, 1971; Radda & Vanderkooi, 1972; Azzi, 1975).

Materials and Methods

Materials

Bovine serum albumin (crystalline), lysophosphatidylcholine (egg yolk) and linoleic acid were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. The albumin was defatted as described by Graham *et al.* (1977). Purified phospholipase A (proteinase free) was prepared as described by Graham & Wood (1969). 8-Anilidonaphthalene-1-sulphonate of spectrophotometric quality and *N*-phenyl-1-naphthylamine were from BDH Chemicals, Poole, Dorset, U.K. 8-Anilidonaphthalene-1-sulphonate was used without further purification; *N*-phenyl-1-naphthylamine was recrystallized three times from aqueous methanol and dried *in vacuo* over silica gel. Purified *N*-phenyl-1-naphthylamine had m.p. 61–62°C (lit. 62°C; Vogel, 1967) and ran as a

single spot on t.l.c. with two solvent systems (Delves, 1967).

Microsomal fractions

Male rats (Wistar strain, 150–200g) were from Fisons Pharmaceuticals, Loughborough, Leics., U.K., or from Animal Supply Ltd., Welwyn, Herts., U.K. Male guinea pigs (Hartley strain, 250–400g) were from David Hall, Newchurch, Burton-on-Trent, Staffs., U.K. The animals were starved overnight, killed and liver microsomal fractions prepared with 0.25M-sucrose or 154mM-KCl (Graham & Wood, 1973). The washed microsomal membranes were resuspended, adjusted with the appropriate medium to 20 or 40mg of protein/ml, stored at –20°C and used within 24h. Microsomal protein was determined with a biuret reagent (Graham & Wood, 1974). The UDP-glucuronyltransferase activities (assayed with 4mM-UDP-glucuronate) at 37°C fell within the ranges reported for similar preparations (Graham & Wood, 1973).

Perturbation methods

Microsomal membranes (final concn. 10mg of protein/ml) were digested with phospholipase A (10µg/mg of microsomal protein) for 5min at 20°C in the presence of 15mM-Tris/HCl buffer, pH 8.0, and 2.5mM-CaCl₂. Phospholipid hydrolysis was stopped by the addition of EDTA (final concn. 10mM).

Lysophosphatides and fatty acids produced by phospholipase A were removed by washing the modified membranes with a solution of defatted albumin (Graham *et al.*, 1977).

Microsomal membranes (final concn. 10mg of protein/ml) were treated for 10min at 20°C with lysophosphatidylcholine (0.3µmol/mg of protein) or Triton X-100 (0.1%) as described by Graham & Wood (1974). Treatment with linoleic acid (0.3µmol/mg of protein) was for 30min at 27°C.

UDP-glucuronyltransferase assays

Transferase activities of native and perturbed microsomal membranes were determined with 0.6mM-*p*-nitrophenol and 4mM- or 25mM-UDP-glucuronate by the method of Graham & Wood (1973) with the following improvements. The incubation volume during assay was increased to 0.8ml and, at seven appropriate times, 0.1ml portions were removed and added to 2ml of cold 0.1M-trichloroacetic acid. Activity (nmol of *p*-nitrophenol glucuronidated/min per mg of microsomal protein) was calculated from initial reaction velocity.

For Arrhenius plots activities were determined at various temperatures between 5 and 50°C with amounts of microsomal protein in the incubation

mixtures varying from 0.1 to 8 mg. The concentrations of KCl or sucrose were constant over the entire temperature range (38.5 and 62.5 mM respectively). Initial velocity varied linearly with microsomal protein concentration at 11 and 35°C. The pH of the buffer (50 mM-KH₂PO₄/KOH, pH 7.1 at room temperature, 17°C) used for assay varied from 7.3 at 0°C to 6.5 at 50°C.

Phospholipids and dispersions

Microsomal fractions (approx. 160 mg of protein) were freeze-dried and total lipids extracted with 3 × 40 ml of chloroform/methanol (2:1, v/v). The combined extracts were washed with water (24 ml) and the bottom phase was dried *in vacuo* at room temperature. The phospholipid fraction was isolated by column chromatography (Graham & Wood, 1969).

Dispersions of the phospholipid mixtures were prepared by ultrasonication (Attwood *et al.*, 1971). Phospholipid (3.75 mg) was dissolved in diethyl ether (approx. 5 ml) and water (3 ml) added. Ether was removed with a stream of N₂ and the aqueous suspension sonicated at 21 kHz for ten 1 min periods alternating with periods of cooling on ice.

Fluorescence probe techniques

The procedures used are based on those of Overath & Träuble (1973). Microsomal membranes were added to freshly prepared solutions of *N*-phenyl-1-naphthylamine [in 0.25 M-NaCl containing 1% (v/v) methanol] or 8-anilino-naphthalene-1-sulphonate (in 0.1 M-KH₂PO₄/KOH buffer, pH 7.1) to give these final concentrations; microsomal protein, 0.1 mg/ml; *N*-phenyl-1-naphthylamine, 10 μM; 8-anilino-naphthalene-1-sulphonate, 20 μM. Portions of the phospho-

lipid dispersions were added to solutions of *N*-phenyl-1-naphthylamine or 8-anilino-naphthalene-1-sulphonate to give the same probe concentrations and a final phospholipid concentration of approx. 235 μM. These mixtures were incubated for 15 min at 30°C in the dark and transferred to a thermostatically controlled cell (1 cm path-length) in a Perkin-Elmer MPF-3 spectrofluorimeter. The temperature was increased from 5 to 50°C at about 0.5°C/min and the mixtures were irradiated continuously at the observed excitation $\lambda_{\max}$ for *N*-phenyl-1-naphthylamine (347–352 nm) or at 380 nm for 8-anilino-naphthalene-1-sulphonate. Emission intensity was recorded continuously at the observed emission $\lambda_{\max}$ for *N*-phenyl-1-naphthylamine (410–420 nm) or at 470 nm for 8-anilino-naphthalene-1-sulphonate. In several experiments fluorimetric measurements were made while mixtures cooled from 50°C.

The binding of *N*-phenyl-1-naphthylamine to microsomal-membrane phospholipids was investigated as described by Bashford *et al.* (1976). Rat microsomal membranes prepared with KCl (0.1–0.67 mg of protein/ml) were titrated with 10 μM-*N*-phenyl-1-naphthylamine and the 'limiting fluorescence' at infinite membrane concentration was estimated from a double-reciprocal plot. Then, *N*-phenyl-1-naphthylamine (6–22 μM) was titrated at constant membrane concentration (0.5 mg of protein/ml) and the number of binding sites (n) and dissociation constant (K_d) for the interaction were determined by Scatchard (1949) analysis (Bashford *et al.*, 1976).

Results and Discussion

Fig. 1 shows typical Arrhenius plots for UDP-glucuronyltransferase activity in liver microsomal

Fig. 1. Arrhenius plots of UDP-glucuronyltransferase activity of native microsomal membranes. Transferase activity (nmol/min per mg of microsomal protein) was determined with (●) 4 mM- or (○) 25 mM-UDP-glucuronate. (a) Guinea-pig microsomal fraction prepared with 154 mM-KCl; (b) guinea-pig microsomal fraction prepared with 0.25 M-sucrose; (c) rat microsomal fraction prepared with 154 mM-KCl; (d) rat microsomal fraction prepared with 0.25 M-sucrose.

Table 1. *Arrhenius-plot transition parameters of microsomal UDP-glucuronyltransferase activity*

Apparent activation energies (E_a) and transition temperatures (T_c) were estimated from Arrhenius plots of the UDP-glucuronyltransferase activity of the various native microsomal fractions (see Fig. 1). The microsomal membranes were prepared and stored at -20°C in 154 mM-KCl or 0.25 M-sucrose and used within 24 h. The degree of latency of the transferase in these preparations is assessed as the percentage increase in activity (assayed with 4 mM-UDP-glucuronate) produced by perturbation with Triton X-100, sodium deoxycholate or phospholipase A (data from Graham & Wood, 1973).

Animal	Membranes prepared with	Degree of latency (%)	Concn. of UDP-glucuronate (mM)	T_c ($^\circ\text{C}$)	E_a (kJ/mol)		All values of T measured
					$T < T_c$	$T > T_c$	
Guinea pig	154 mM-KCl	0–60	4	—	—	—	56–63
			25	—	—	—	59
Guinea pig	0.25 M-Sucrose	130–500	4	20	21–39	70–72	—
			25	23	39	68	—
Rat	154 mM-KCl	330–500	4	20–22	24–39	49–70	—
			25	22	24	57	—
Rat	0.25 M-Sucrose	500–740	4	22–25	25–28	60–65	—
			25	27	28	84	—

fractions from guinea pig and rat prepared with 154 mM-KCl or 0.25 M-sucrose. The transferase of our KCl-prepared guinea-pig membranes, which is highly active and almost free from latency (Graham & Wood, 1972, 1973; Table 1), exhibited linear plots from 5 to 40°C (Fig. 1a). Above 40°C thermal inactivation of the enzyme was observed. Values of E_a varied in different microsomal preparations from 56 to 63 kJ/mol (Table 1). The remaining microsomal preparations, which have lower transferase activities and different degrees of transferase latency (Graham & Wood, 1972, 1973; Table 1), gave Arrhenius plots (Figs. 1b–1d) with two linear regions intersecting at a sharp transition point (T_c). Values of T_c and E_a (above and below T_c) varied somewhat in different microsomal preparations, but this did not appear to be related to animal species or to the way of preparing the membranes (Table 1).

Saturating concentrations of substrates were not used in these experiments. However, similar Arrhenius plots were obtained by using 4 mM- and 25 mM-UDP-glucuronate (Fig. 1) and values of E_a and T_c at the two sugar nucleotide concentrations were indistinguishable (Table 1). Latent UDP-glucuronyltransferase is activated by maintaining the microsomal preparations at 37°C (Leuders & Kuff, 1967; Winsnes, 1969). Any such thermal activation of latent enzyme clearly would affect the interpretation of our results (Figs. 1b–1d). The activity of sucrose-prepared rat microsomal membranes was determined at 12°C (below T_c). The membrane suspension was incubated at 35°C (above T_c) for 20 min, cooled to 12°C and transferase activity re-determined immediately. The two activities measured at 12°C were identical, showing that the change in properties of the latent enzyme at or above T_c is rapidly revers-

ible and that irreversible thermal activation does not occur. The pH of the buffer used for assay decreased with increase in temperature (see the Materials and Methods section). However, pH-activity curves from data obtained at 12 and 35°C showed that transferase activity gradually increased when the pH was raised from 6 to 10, and we conclude that this variation of assay pH with temperature also is not responsible for the biphasic nature of Arrhenius plots of latent UDP-glucuronyltransferase activity.

Transferase latency is removed by modifying membrane phospholipids with phospholipase A (Hänninen & Puukka, 1971; Vessey & Zakim, 1971; Graham & Wood, 1973, 1974; Graham *et al.*, 1974a,b) or by treatment with surface-active agents (Dutton & Burchell, 1977). When sucrose-prepared rat liver microsomal fractions, which contain extremely latent transferase activity (Graham & Wood, 1973; Table 1), were treated with perturbants under conditions causing maximum activation (Graham & Wood, 1974), Arrhenius plots of enzyme activity were quite different from those exhibited by native membranes (Fig. 2). Treatment with phospholipase A, which hydrolyses about one-third of the microsomal phospholipids (mainly phosphatidylcholine and phosphatidylethanolamine), activated UDP-glucuronyltransferase over the entire temperature range. The biphasic nature of the Arrhenius plot was abolished and E_a over the whole temperature range is similar to that of latent microsomal enzyme below T_c (Fig. 2a). Activation by phospholipase A is due to the surface-active products of its catalytic action (Graham & Wood, 1974), and two of the major products, lysophosphatidylcholine and linoleic acid, are effective activators (Hänninen & Puukka, 1971; Graham & Wood, 1974). When microsomal mem-

Fig. 2. Arrhenius plots of UDP-glucuronyltransferase activity of rat microsomal membranes treated with membrane perturbants

Rat microsomal membranes prepared with 0.25M-sucrose were treated with the various perturbants as described in the Materials and Methods section. Transferase activity (nmol/min per mg of microsomal protein) was determined with 4mM-UDP-glucuronate after perturbation (●) and at a temperature near 30°C before perturbation (○). (a) Treated with phospholipase A (E_a 32kJ/mol); (b) treated with linoleic acid (E_a 34kJ/mol); (c) treated with lysophosphatidylcholine (T_c 27°C; E_a below T_c , 24kJ/mol; E_a above T_c , 37kJ/mol); (d) treated with Triton X-100 (T_c , 23°C; E_a below T_c , 23kJ/mol; E_a above T_c , 41kJ/mol).

branes were treated with these compounds at concentrations similar to those produced by the phospholipase, linoleic acid abolished the biphasic shape of the Arrhenius plot (Fig. 2b), and the lysophosphatide, although it did not completely eliminate the transition, greatly decreased E_a above T_c (Fig. 2c). With Triton X-100 a change of slope still occurred at the same temperature (23°C) as with native membranes, but again E_a above T_c was lowered considerably (Fig. 2d). Activation of latent UDP-glucuronyltransferase by phospholipase A and its hydrolysis products is reversed by defatted albumin, which binds and removes the stimulatory compounds (Graham & Wood, 1974; Graham *et al.*, 1977). This was confirmed in the present work (Fig. 3). However, albumin washing after phospholipase treatment did not restore the biphasic response of rat liver transferase to temperature; no transition was detected in the Arrhenius plot between 4 and 30°C, and above 30°C thermal inactivation occurred (Fig. 3). This might indicate that the effect of phospholipase A on Arrhenius plots of latent enzyme activity is not solely due to surface-active products. However, even thorough washing of the modified membranes does not remove these stimulatory compounds completely (Graham *et al.*, 1977) and the small amounts remaining in the membranes might still affect the transferase's response to temperature.

The data of Figs. 1 and 2 are similar to those of Eletr *et al.* (1973) and Norling & Hänninen (1974). In addition, our data also show that the perturbants activate latent UDP-glucuronyltransferase at all temperatures between 5 and 50°C and that the ther-

Fig. 3. Arrhenius plot of UDP-glucuronyltransferase activity of partially delipidated rat microsomal membranes

Rat microsomal membranes prepared with 0.25M-sucrose were delipidated by treatment with phospholipase A followed by washing with a solution of albumin (see the Materials and Methods section). The transferase activities (nmol/min per mg of microsomal protein) of partially delipidated membranes (●), and at 35°C of phospholipase-treated (□) and native (○) membranes, were determined by using 4mM-UDP-glucuronate. E_a between 4 and 30°C was 40kJ/mol.

mal transition in transferase activity occurs over a narrow temperature range, is rapidly reversible and is associated only with latent microsomal enzyme. Arrhenius plots for several membrane-bound enzymes show sharp transitions or break-points between two linear regions (see the introduction), but latent transferase is one of only a few whose E_a value above T_c is greater than that below T_c . Release of the transferase from latency by perturbants that interact primarily with the lipid component of membranes abolishes the thermal transition or greatly decreases the difference between the two values of E_a , suggesting that the thermotropic change in the enzyme's properties is associated with a structural alteration of the membrane phospholipid bilayer. Eletr *et al.* (1973) found a break-point in Arrhenius plots of UDP-glucuronyltransferase ac-

tivity in sucrose-prepared guinea-pig microsomal fractions at 19°C, i.e. close to our values of T_c . Their report of a second break-point at 32°C was not confirmed in our more extensive studies. These workers showed that the 19°C transition coincided with an abrupt increase in membrane-lipid fluidity. We have used lipophilic fluorescent probes to study any thermotropic change of phospholipid structure in the region of T_c .

When the fluorescent probes 8-anilinonaphthalene-1-sulphonate and *N*-phenyl-1-naphthylamine were incorporated into microsomal membranes, both showed the enhanced fluorescence and spectral shifts typical of these compounds in a lipid membrane or other environment of low polarity (Radda & Vanderkooi, 1972; Träuble & Overath, 1973; Azzi, 1975). With KCl-prepared rat microsomal membranes the

Fig. 4. Temperature-dependence of the fluorescence of 8-anilinonaphthalene-1-sulphonate bound to rat microsomal membranes and phospholipids

Temperature-dependence of 8-anilinonaphthalene-1-sulphonate fluorescence with the probe incorporated into rat microsomal membranes prepared with 154 mM-KCl (Fig. 4a) and into phospholipids extracted from these membranes (Fig. 4b) was measured as described in the Materials and Methods section.

Fig. 5. Temperature-dependence of the fluorescence of *N*-phenyl-1-naphthylamine bound to rat microsomal membranes and phospholipids

The temperature-dependence of *N*-phenyl-1-naphthylamine fluorescence with the probe incorporated into rat microsomal membranes prepared with 154 mM-KCl (Fig. 5a) and into phospholipids extracted from these membranes (Fig. 5b) was measured as described in the Materials and Methods section.

Table 2. *Thermotropic structural transitions detected by N-phenyl-1-naphthylamine fluorescence*

N-Phenyl-1-naphthylamine was incorporated into the various microsomal preparations and dispersions of phospholipids extracted from membranes prepared with 154mM-KCl as described in the Materials and Methods section. Transition midpoints (T_m) and transition widths (ΔT) were estimated from plots of fluorescence intensity against temperature (see Fig. 5) by the method of Thilo & Overath (1976).

	Major transition		Subsidiary transitions		
	T_m (°C)	ΔT (°C)	T_m (°C)		
Guinea-pig membranes, 154mM-KCl	22–24	1–3	29–31.5	15.5	11–12
Guinea-pig membranes, 0.25M-sucrose	25	4	—	—	12
Rat membranes, 154mM-KCl	21	3	30.5	15	12
Rat membranes, 0.25M-sucrose	22	4	31.5	15.5	12
Guinea-pig phospholipids	22.5	4	31.5	15	12
Rat phospholipids	23.5	3	—	—	12

intensity of 8-anilino-naphthalene-1-sulphonate fluorescence declined with increasing temperature in a monotonic fashion (Fig. 4a). With *N*-phenyl-1-naphthylamine the plot of fluorescence intensity against temperature (Fig. 5a) showed a number of sigmoid transitions of the type attributed to phospholipid phase changes (Overath & Träuble, 1973; Träuble & Overath, 1973). The most prominent is centred at 21°C (T_m) with a transition width (ΔT) of 3°C (Table 2). A second small transition occurred at 30.5°C and slight irregularities in the curve might suggest very small transitions near 12 and 15°C. After a heating run when the *N*-phenyl-1-naphthylamine-labelled membranes were cooled from 45°C the transitions at 30.5 and 21°C were observed again, showing that the structural changes that they signify are reversible. The cooling curve was not superimposable on the heating curve below 25°C, but this probably is not related to changes in phospholipid structure. This phenomenon has been recorded with *N*-phenyl-1-naphthylamine (Overath & Träuble, 1973) and seems to be caused by photo-decomposition of the fluorophore (G. K. Radda, personal communication). T.l.c. (Delves, 1967) of a solution of *N*-phenyl-1-naphthylamine in methanol after irradiation in the spectrofluorimeter cell under the conditions of a heating run revealed the presence of trace amounts of at least four components not present in a fresh solution of the probe. This suggests that during these experiments a small amount of decomposition occurred, and in subsequent work only heating curves were recorded. Plots of fluorescence versus temperature with 8-anilino-naphthalene-1-sulphonate and *N*-phenyl-1-naphthylamine for all four types of microsomal preparation were very similar to those given by rat KCl-prepared membranes; the transition parameters obtained with *N*-phenyl-1-naphthylamine are quoted in Table 2.

8-Anilino-naphthalene-1-sulphonate incorporated into dispersions of microsomal phospholipids did not reveal any thermotropic alteration to the structure of the bilayers (Fig. 4b); the response of its fluore-

scence under our experimental conditions in both membranes and lipid bilayers was that expected of a fluorophore dissolved in an ideal liquid (Radda, 1971). Curves of fluorescence intensity against temperature for *N*-phenyl-1-naphthylamine-labelled dispersions of phospholipid extracted from rat KCl-prepared microsomal fractions showed one major transition, with T_m at 23.5°C (Fig. 5b). It was more pronounced than the corresponding transition in intact membranes, and the membrane transition at 30.5°C (Fig. 4a) was barely detectable with the phospholipid dispersion. A similar result was obtained with dispersions of guinea-pig phospholipids extracted from KCl-prepared membranes, but the 31.5°C transition (Table 2) was similar in size to that in intact microsomal membranes. We conclude that the major fluorescence transition centred at 21–25°C is a characteristic of the phospholipid bilayer of the microsomal membranes. It is also noteworthy that the temperature of the intersection of the two linear regions (T_c) of Arrhenius plots for latent microsomal UDP-glucuronyltransferase (Table 1) coincides with the mid-point of the main fluorescence transition detected by *N*-phenyl-1-naphthylamine.

8-Anilino-naphthalene-1-sulphonate is amphiphilic and located near the polar/apolar interface of membrane phospholipid bilayers with limited penetration of its chromophore into the hydrocarbon core. On the other hand, *N*-phenyl-1-naphthylamine is hydrophobic and bound within the hydrocarbon core (Radda & Vanderkooi, 1972; Azzi, 1975). Thus the structural transitions detected in microsomal membranes and phospholipid dispersions by *N*-phenyl-1-naphthylamine, but not by 8-anilino-naphthalene-1-sulphonate, particularly that at 21–25°C, which appears to affect latent microsomal UDP-glucuronyltransferase, are changes in the physical state or organization of acyl chains deep within the bilayers. Several studies of thermotropism in microsomal membranes have used fluorescent or nitroxide moieties attached to different carbon atoms

of the stearic acid acyl chain whose location in the membrane can be predicted confidently. Whereas probes with a reporter group at C-12 or C-13 detected a transition near 22°C (Eletr & Inesi, 1972; Inesi *et al.*, 1973; Grisham & Barnett, 1973; Raison & McMurchie, 1974; Charnock & Bashford, 1975) a reporter group at C-5 was unable to detect any structural change (Stier & Sackmann, 1973). A C-5 reporter group, like 8-anilidonaphthalene-1-sulphonate, is located near the polar head-group region of the bilayer, whereas a C-12 or C-13 group, like *N*-phenyl-1-naphthylamine, is buried in the hydrocarbon phase. Our findings are consistent with these results.

Only latent microsomal UDP-glucuronyltransferase displays biphasic Arrhenius plots (Fig. 1). The perturbants used interact with the phospholipid component of microsomal membranes, increasing permeability (Fiehn & Hasselbach, 1970; Kreibich *et al.*, 1973; Nilsson & Dallner, 1977), and are considered to remove transferase latency by increasing access of substrates to deeply buried sites of glucuronidation (see the introduction). Perturbation activated latent enzyme at all temperatures from 5 to 50°C (Fig. 2); we conclude that in these native membranes, permeability to substrates is the rate-controlling step of glucuronidation over the whole temperature range

and that the values of E_a are characteristic of a permeation process. Perturbants lowered E_a above T_c , so abolishing or greatly diminishing the biphasic nature of Arrhenius plots (Fig. 2), and we suggest that the change in the properties of the latent system is due to a permeability change caused by a thermal alteration of the hydrocarbon core of the membrane phospholipid bilayer. It is obvious, however, that this thermotropic increase in permeability to the enzyme's substrates is quite small compared with that induced by perturbation of the microsomal phospholipid bilayer. The UDP-glucuronyltransferase of guinea-pig microsomal fractions as we have prepared them with KCl is highly active and almost free from latency (Table 1). It appears to be located at or near the membrane surface almost unrestricted by the permeability barrier (see the introduction), and therefore would not be influenced by such a change of permeability. This enzyme form has a linear Arrhenius plot (Fig. 1a) and its higher value of E_a might be a parameter of the catalytic reaction itself.

Spectroscopic evidence for a thermotropic structural change in microsomal phospholipids near 20–25°C is well documented (see the introduction). However, the nature of this phospholipid phase transition is rather poorly characterized. Zakim's group considered that the break in their Arrhenius

Fig. 6. Analysis of the interaction of *N*-phenyl-1-naphthylamine with rat microsomal membranes at temperatures above and below T_m

Fluorescence intensities of *N*-phenyl-1-naphthylamine (NPN) bound to rat microsomal membranes prepared with 154 mM-KCl were measured as described in the Materials and Methods section and the results shown as double-reciprocal plots (Fig. a) and Scatchard (1949) plots (Fig. b). Titrations were at (●) 16 and (○) 28°C. From three separate experiments the following values for the equilibrium parameters were evaluated. At 16°C: n_1 (primary sites), 13–22; K_{d1} , 0.45–0.46 μM; n_2 (secondary sites), 18–19; K_{d2} , 2.88–3.31 μM; at 28°C: n_1 , 13–22; K_{d1} , 0.63–0.70 μM; n_2 , 18–19; K_{d2} , 3.72–3.91 μM.

plots at 19°C (Eletr *et al.*, 1973) or 16°C (Zakim & Vessey, 1975) was associated with an abrupt increase in membrane fluidity at 19°C (Eletr *et al.*, 1973). Zakim & Vessey (1975) concluded that the bulk of the microsomal lipids had undergone a crystalline/liquid-crystalline transition. However, calorimetric measurements show that 'melting' of these lipid acyl chains occurs at below 0°C (Blazyk & Steim, 1972; Martonosi, 1974; Mabrey *et al.*, 1977). To investigate the nature of the transition in microsomal phospholipids that we observe between 21 and 25°C (Fig. 5, Table 2) we have studied the binding of *N*-phenyl-1-naphthylamine to microsomal membranes at temperatures above and below T_m by Scatchard (1949) analysis.

Fig. 6 gives typical results for the interaction of *N*-phenyl-1-naphthylamine with KCl-prepared rat microsomal membranes at two temperatures. Two types of binding site are identified both above and below the mid-point of the transition, and, when the temperature is increased through T_m , K_d for probe binding at both types of site is increased, with no significant change in the numbers of the sites. These are not the sorts of change in equilibrium parameters expected on traversing a crystalline/liquid-crystalline transition. Träuble & Overath (1973) using bilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine showed that 'melting' of acyl chains is accompanied by changes in the number of probe-binding sites and very little change in K_d . Bashford *et al.* (1976) also observed that increase in temperature through a critical range caused changes in binding characteristics of *N*-phenyl-1-naphthylamine to chromaffin-granule membranes very similar to those reported here and concluded that at or below T_m there was an increase in the order of the lipid phase caused by 'phase separation' or clustering of the membrane phospholipids into discrete domains. We conclude therefore that the thermotropic change in permeability, which affects the properties of latent UDP-glucuronyltransferase, is caused by a subtle increase in fluidity of the enzyme's phospholipid environment, which is possibly a deeply buried hydrocarbon domain with which the latent enzyme is closely associated.

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