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THE EFFECTS OF SULFHYDRYL REACTING AGENTS ON HEPATIC UDP-GLUCURONYLTRANSFERASE *IN VITRO*

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SUMMARY

The effects of *N*-ethylmaleimide, iodoacetamide and *p*-chloromercuribenzoate on both "native" and activated mouse- and rat-liver glucuronyltransferase (UDP-glucuronate glucuronyltransferase (acceptor unspecific), EC 2.4.1.17) were studied. The following five acceptor substrates were used: *p*-nitrophenol, *o*-aminophenol, phenolphthalein, 4-methylumbelliferone and bilirubin.

Contrary to earlier results, the present report indicates that the active site of glucuronyltransferase is not dependent on sulfhydryl groups for catalysis. These apparent discrepancies are explained by the change in the UDP-glucuronate saturation characteristics of glucuronyltransferase when treated with sulfhydryl reacting agents. At low concentrations of UDP-glucuronate complete inhibition was achieved with *N*-ethylmaleimide, whereas at high concentration of substrate activation of *p*-nitrophenol glucuronyltransferase by *N*-ethylmaleimide was observed.

While the activation by Triton X-100 of glucuronyltransferase was little affected by pretreatment of the enzyme suspension with thiol blocking agents, the activating effect of UDP-*N*-acetylglucosamine was very sensitive towards such treatment. 1 mM *N*-ethylmaleimide, 30 mM iodoacetamide or 0.5 mM *p*-chloromercuribenzoate completely desensitized *p*-nitrophenol glucuronyltransferase towards 2 mM UDP-*N*-acetylglucosamine. The desensitization by *N*-ethylmaleimide was completely irreversible, while the effect of iodoacetamide was partly reversed and that of *p*-chloromercuribenzoate completely reversed by dithiothreitol.

The possible mechanisms underlying the activation of glucuronyltransferase by UDP-*N*-acetylglucosamine *in vitro* are discussed.

INTRODUCTION

Hepatic microsomal glucuronyltransferase (UDP-glucuronate glucuronyltransferase (acceptor unspecific), EC 2.4.1.17) activity is enhanced severalfold *in vitro* by addition of UDP-*N*-acetylglucosamine[1-3] or low concentrations of detergents[2,4-6] in the incubation mixture. The mechanism(s) underlying this activation *in vitro* is poorly understood.

In an earlier study from this laboratory[2] it was shown that the effects of Triton X-100 and UDP-*N*-acetylglucosamine were non-additive towards each other. From kinetic experiments and lack of evidence for the existence of an inhibitor, the hypothesis was put forward that an "unmasking" of active sites could explain the activation observed *in vitro*[2]. The effect of UDP-*N*-acetylglucosamine was suggested to be caused by an allosteric mechanism, whereas the effect of detergents more probably would be of an unspecific nature[2], since several microsomal enzymes are activated similarly by such agents[7–10].

In the hope to gain further information about the mechanism(s) whereby UDP-*N*-acetylglucosamine and detergents activate glucuronyltransferase, a study of the effects of sulfhydryl-reacting agents on the enzyme activity was performed. In contrast to the activation by Triton X-100 and digitonin, the activating effect of UDP-*N*-acetylglucosamine was found to be dependent on intact sulfhydryl groups. The present study also indicates that the active site of glucuronyltransferase is not dependent on sulfhydryl groups in contrast to an earlier report[11].

MATERIALS AND METHODS

The animals used routinely (Figs. 1–5) were male NMRI/BOM mice (30–40 g weight). Female NMRI/BOM mice (20–30 g weight) and female Wistar rats (150–250 g weight) were used in a comparative study (Table I). Liver homogenates (in 0.154 M KCl solution) were centrifuged at 2000 × *g* for 15 min, and the supernatant was used for enzyme assay.

Acceptor substrates (final concentration 0.5 mM) were *p*-nitrophenol, *o*-aminophenol, phenolphthalein, 4-methylumbelliferone and bilirubin. Where otherwise is not stated the UDP-glucuronate concentration was 2.0 mM and the pH of the incubation mixture 7.4 (75 mM Tris–maleate buffer). Enzyme activators and their final concentrations were: 2 mM UDP-*N*-acetylglucosamine; 0.05% (w/v) Triton X-100 when *p*-nitrophenol, phenolphthalein and 4-methylumbelliferone were acceptors; 0.075% (w/v) Triton X-100 with bilirubin as acceptor and 0.2% (w/v) digitonin with *o*-aminophenol as acceptor. With *p*-nitrophenol as substrate tissue suspension corresponding to 15 mg wet weight liver was used. Incubation mixtures were kept in stoppered glass tubes and incubated aerobically at 37° for 15 min when UDP-*N*-acetylglucosamine or Triton X-100 were included in the assay. Non-activated enzyme was routinely incubated for 30 min, but in the kinetic studies (Fig. 4) 15 min incubation time was used. For more detailed description of the assay methods see ref. 2.

Solutions of *N*-ethylmaleimide (Fluka) and dithiothreitol (Calbiochem) were made fresh each day, whereas stock solutions of iodoacetamide (Fluka) and *p*-chloromercuribenzoate (Sigma) were kept frozen.

RESULTS

The effect of N-ethylmaleimide, iodoacetamide and p-chloromercuribenzoate

As shown in Fig. 1 "native" mouse-liver *p*-nitrophenol glucuronyltransferase (with 2 mM of UDP-glucuronate in the incubation mixture) was little affected by lower concentrations of *N*-ethylmaleimide whether the enzyme suspension was

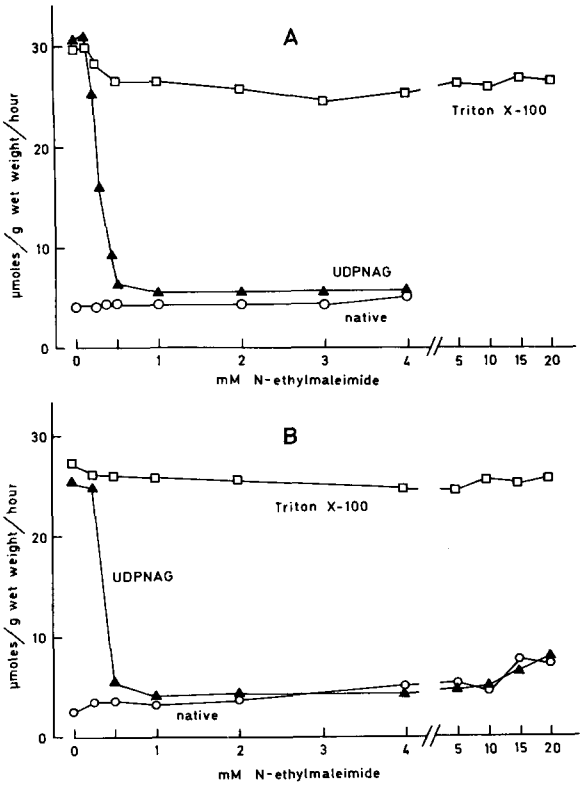


Fig. 1. Mouse-liver *p*-nitrophenol glucuronyltransferase was assayed in presence of UDP-*N*-acetylglucosamine (UDPNAG) and Triton X-100 as well as without these agents (native). The results of an experiment where enzyme suspension was added as the last component to the incubation mixture (A) are compared with those obtained when enzyme suspension was preincubated with varying concentrations of *N*-ethylmaleimide for 15 min at 4° before addition of substrates and activators (B). The final concentrations of *N*-ethylmaleimide are given along the abscissa. The concentrations of *N*-ethylmaleimide during preincubation were 2.5-fold higher.

pretreated with the alkylating agent (Fig. 1B) or not (Fig. 1A). However, with 15–20 mM *N*-ethylmaleimide present three-fold increased enzyme activity was found (Fig. 1B). A similar degree of activation was achieved by treatment with 1 mM *p*-chloromercuribenzoate, whereas this reagent inhibited the "native" enzyme at concentrations up to 0.4 mM (Fig. 2B). A slight increase in activity of "native" glucuronyltransferase at higher concentrations of iodoacetamide was also found (Fig. 2A).

In contrast to "native" enzyme, glucuronyltransferase activated by 2 mM UDP-*N*-acetylglucosamine was very sensitive towards the effect of sulfhydryl-reacting agents. At increasing concentrations of both *N*-ethylmaleimide, iodoacetamide and *p*-chloromercuribenzoate the activating effect of UDP-*N*-acetylglucosamine gradually decreased, and the enzyme activity approached that of the "native" enzyme at 1 mM *N*-ethylmaleimide (Figs. 1A and 1B), 30 mM iodoacetamide (Fig. 2A) and 0.5 mM *p*-chloromercuribenzoate (Fig. 2B). The same activation as seen with "native"

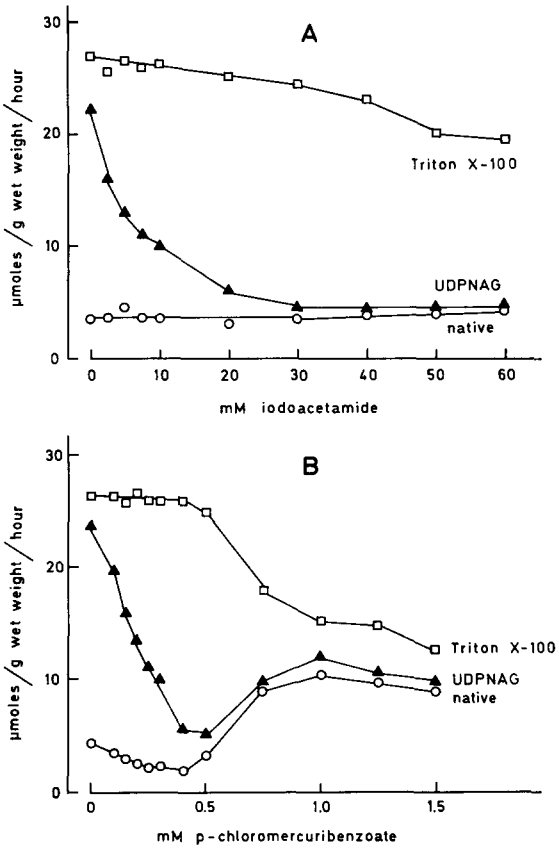


Fig. 2. Mouse-liver suspension was preincubated for 15 min at 4° with varying concentrations of iodoacetamide (A) and *p*-chloromercuribenzoate (B), before addition of substrates and activators. The concentrations of iodoacetamide and *p*-chloromercuribenzoate during preincubation were 2.5-fold higher than the final concentrations given in the figure. *p*-Nitrophenol glucuronyltransferase activities were assayed in the presence of UDP-*N*-acetylglucosamine (UDPNAG) and Triton X-100 as well as without these agents (native).

enzyme was seen at higher concentrations of *N*-ethylmaleimide (Fig. 1B) and *p*-chloromercuribenzoate (Fig. 2B).

p-Nitrophenol glucuronyltransferase activated by Triton X-100 on the other hand, was not much affected by sulfhydryl-reacting agents. *N*-ethylmaleimide inhibited slightly at lower concentrations, but at 20 mM the activity was still 90–95% of that without *N*-ethylmaleimide even when the enzyme suspension was pretreated with the alkylating agent (Figs. 1A and 1B). The activating effect of Triton X-100 decreased slowly at increasing concentrations of iodoacetamide, with 73% of the activity remaining at 60 mM iodoacetamide. 1.5 mM *p*-chloromercuribenzoate resulted in 57% inhibition of *p*-nitrophenol glucuronyltransferase when assayed in the presence of Triton X-100. However, in the concentration range 0.1–0.5 mM where the UDP-*N*-acetylglucosamine activation decreased rapidly, the activation by Triton X-100 was little affected by *p*-chloromercuribenzoate.

Since detergents might increase the number of active sites accessible for the substrates[2,4,6] (and perhaps also for *N*-ethylmaleimide), preincubation of liver suspension with both Triton X-100 and *N*-ethylmaleimide, but without substrates should result in blockade of the active site if this involved sulfhydryl-groups. Hence an experiment was performed where enzyme suspension was preincubated for 1 h with 0.125% (w/v) Triton X-100 and 5 mM *N*-ethylmaleimide. After preincubation, substrates and buffer were added (resulting in 2.5-fold dilution), and the enzyme activity was compared with that obtained by adding enzyme suspension to an equivalent incubation mixture just prior to incubation. There was no significant difference between the activities obtained (which were just slightly below that of Triton X-100 activated enzyme without *N*-ethylmaleimide), indicating that the active site of *p*-nitrophenol glucuronyltransferase is not dependent on intact sulfhydryl-groups.

Reversal of sulfhydryl blockade by dithiothreitol

When enzyme pretreated with *N*-ethylmaleimide was assayed in the presence of 2 mM UDP-*N*-acetylglucosamine and varying concentrations of dithiothreitol, no reversal of the desensitization towards UDP-*N*-acetylglucosamine was observed as expected with this alkylating agent (Fig. 3A). Enzyme pretreated with iodoacetamide and assayed in presence of UDP-*N*-acetylglucosamine exhibited slightly increasing activity at increasing concentrations of dithiothreitol, though at 20 mM

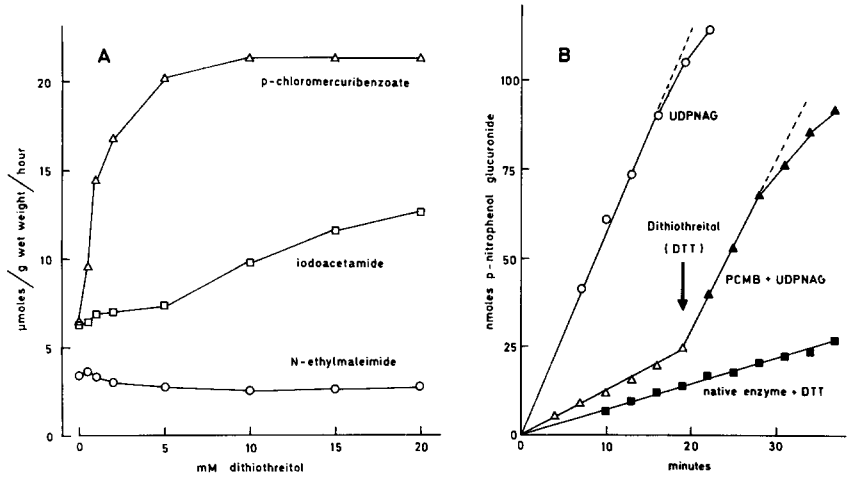


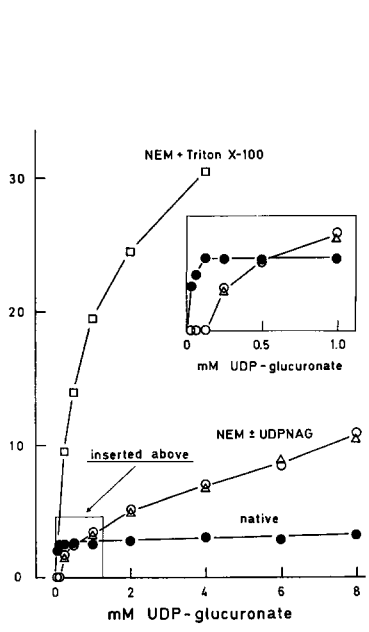
Fig. 3. (A) Mouse-liver suspension was preincubated 15 min at 4° with 5 mM *N*-ethylmaleimide, 100 mM iodoacetamide and 2.5 mM *p*-chloromercuribenzoate, respectively, before addition of *p*-nitrophenol, UDP-glucuronate, UDP-*N*-acetylglucosamine and varying concentrations of dithiothreitol. The concentrations of the sulfhydryl reacting agents during preincubation were reduced 5-fold upon addition of the other reagents. (B) Several test-tubes containing mouse-liver suspension were incubated with *p*-nitrophenol and UDP-glucuronate at 37° for varying lengths of time as indicated. One series of tubes contained UDP-*N*-acetylglucosamine (UDPNAG). A second series contained both UDP-*N*-acetylglucosamine and 0.5 mM *p*-chloromercuribenzoate (PCMB + UDPNAG). The first 6 incubation mixtures in this last series reacted without dithiothreitol, while the last 6 continued the reaction after addition of dithiothreitol to a final concentration of 10 mM at time 19 min. A third series of incubation mixtures contained enzyme suspension without activator, but with 10 mM dithiothreitol (native + DTT).

dithiothreitol the activity was still far below that of the UDP-*N*-acetylglucosamine activated enzyme (22 μmoles/g per h). The desensitization of glucuronyltransferase towards UDP-*N*-acetylglucosamine by *p*-chloromercuribenzoate was, however, completely reversed by 10 mM dithiothreitol (Fig. 3A).

Fig. 3B illustrates that the *p*-chloromercuribenzoate inhibition of glucuronyltransferase with UDP-*N*-acetylglucosamine present was reversed immediately and totally when 10 mM dithiothreitol was added to the incubation mixtures. It is also seen that linearity of the reaction with time was preserved for 15 min with UDP-*N*-acetylglucosamine activated enzyme and at least 30 min with "native" enzyme.

The effect of varying UDP-glucuronate concentrations

The kinetics of "native" enzyme and that pretreated with *N*-ethylmaleimide revealed obvious differences as shown in Fig. 4. At concentrations of UDP-glucuronate in the range 0.03–0.125 mM "native" enzyme exhibited 40–80% of the highest activity recorded (8 mM substrate), whereas complete inhibition of the *N*-ethylmaleimide pretreated enzyme was revealed at these low substrate concentrations. At UDP-glucuronate concentrations above 2 mM where only a very slight increase



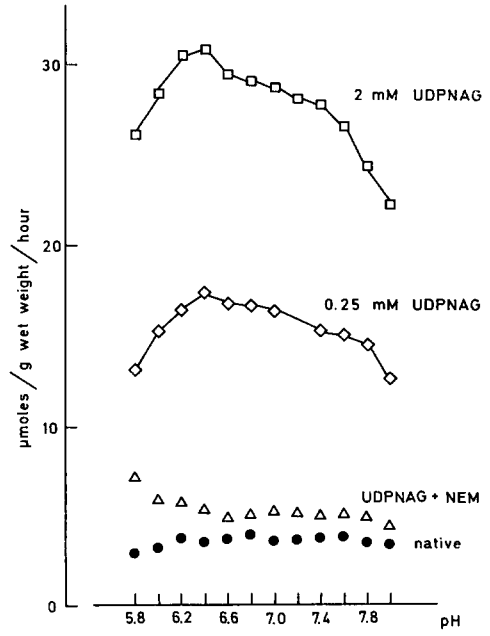


Fig. 4. Mouse-liver suspension was incubated with *p*-nitrophenol (0.5 mM) and UDP-glucuronate concentrations in the range 0.03–8.0 mM. Results with "native" enzyme are compared with those obtained with enzyme pretreated with 5 mM *N*-ethylmaleimide (final concentration 2 mM) and assayed both without and with UDP-*N*-acetylglucosamine (NEM ± UDPNAG) as well as with Triton X-100.

Fig. 5. Mouse-liver suspension was incubated with *p*-nitrophenol and UDP-glucuronate at varying pH values in the range 5.8–8.0. At pH 7.0 and lower units the buffer was 75 mM maleate, while 75 mM Tris–maleate was used at pH 7.2 and higher units. Incubations were performed without additions to the standard assay (native) and with the following additions: 2 mM UDP-*N*-acetylglucosamine + 2 mM *N*-ethylmaleimide (UDPNAG + NEM), 0.25 mM UDP-*N*-acetylglucosamine and 2.0 mM UDP-*N*-acetylglucosamine.

in "native" enzyme activity was seen, there was a steady activity increase of the *N*-ethylmaleimide pretreated enzyme resulting in three-fold higher activity of the latter compared with the former at 8 mM of substrate.

UDP-*N*-acetylglucosamine had no significant effect on the *N*-ethylmaleimide pretreated enzyme at any of the UDP-glucuronate concentrations tested, whereas Triton X-100 activated strongly in the concentration range 0.25–4.0 mM (Fig. 4). The activating effect of Triton X-100 increases with increasing UDP-glucuronate concentrations as also found earlier[2].

The rather slight change in activity of "native" glucuronyltransferase at varying *N*-ethylmaleimide concentrations seen in Fig. 1 is explained by the use of 2 mM UDP-glucuronate in these experiments.

pH optimum of p-nitrophenol glucuronyltransferase

"Native" enzyme exhibited little change in activity in the pH range 6–8 (Fig. 5). Enzyme activated by UDP-*N*-acetylglucosamine revealed highest activities at pH 6.2–6.6 which is the same as found for detergent activated enzyme earlier[2]. When both 2 mM *N*-ethylmaleimide and 2 mM UDP-*N*-acetylglucosamine were present during assay a pH optimum curve similar to that of "native" enzyme was found, though the activity was somewhat higher especially in the low pH range (Fig. 5).

TABLE I

THE EFFECT OF *N*-ETHYLMALEIMIDE ON MOUSE- AND RAT-LIVER UDP GLUCURONYLTRANSFERASE ASSAYED WITH SEVERAL ACCEPTOR SUBSTRATES

Mouse- and rat-liver suspensions were incubated with five different acceptor substrates as indicated. Incubations were performed without additions to the standard assay as well as with 2 mM *N*-ethylmaleimide (NEM), 2 mM UDP-*N*-acetylglucosamine (UDPNAG), both *N*-ethylmaleimide and UDP-*N*-acetylglucosamine, and optimal detergent concentration with and without *N*-ethylmaleimide. The enzyme activities are given as μmoles/g wet weight liver per h. The values represent the mean of two different experiments. Further details were as described in MATERIALS AND METHODS.

Acceptor Substrate	*Animal source*	*Additions to standard assay solutions:*					
		None	*2 mM NEM*	*2 mM UDPNAG*	*2 mM UDPNAG + 2 mM NEM*	*Detergent*	*Detergent + 2 mM NEM*
p-Nitrophenol	Female mouse	3.10	4.00	27.0	4.20	27.2	25.6
	Male mouse	4.04	4.25	26.6	6.10	32.0	28.6
	Male rat	3.54	4.35	13.2	5.70	15.6	14.5
o-Aminophenol	Female mouse	1.28	0.91	5.45	0.94	5.34	6.28
	Male mouse	2.18	—	5.82	1.45	5.22	7.24
	Female rat	0.52	0.53	1.32	0.50	1.51	0.92
Phenolphthalein	Female mouse	2.03	1.75	13.8	1.16	20.5	17.7
	Male mouse	3.09	—	6.84	2.35	26.2	23.3
	Female rat	1.62	2.24	12.5	1.51	20.2	13.0
4-Methylumbelliferone	Female mouse	7.75	10.5	52.5	10.0	112.0	117.0
	Male mouse	8.50	—	57.5	10.0	110.0	95.0
	Female rat	4.17	5.93	35.7	16.0	107.3	98.5
Bilirubin	Female mouse	0.70	0.41	0.71	0.43	2.90	2.68
	Male mouse	0.39	—	0.42	0.34	1.44	1.60
	Female rat	0.79	0.64	1.16	0.64	2.29	2.24

The effect of N-ethylmaleimide on mouse- and rat-liver glucuronyltransferase assayed with several acceptor substrates

2 mM *N*-ethylmaleimide, when added in the incubation mixtures exhibited essentially identical effect whether *p*-nitrophenol, *o*-aminophenol, phenolphthalein or 4-methylumbelliferone were used as acceptors and whether the enzyme source was female or male mice or female rat (Table I). Glucuronyltransferase was desensitized towards the activating effect of 2 mM UDP-*N*-acetylglucosamine by 2 mM *N*-ethylmaleimide, while the effect on the detergent activated enzyme was negligible (Table I). Digitonin activated *o*-aminophenol and Triton X-100 activated phenolphthalein glucuronyltransferases in rat-liver suspension were, however, somewhat more inhibited by *N*-ethylmaleimide than generally seen with detergent activated enzyme, but typically the nucleotide activated enzymes were considerably more sensitive towards *N*-ethylmaleimide (Table I).

As shown in Table I, bilirubin glucuronyltransferase in mouse-liver suspension was not activated by 2 mM UDP-*N*-acetylglucosamine, whereas Triton X-100 had good effect in agreement with earlier results[2]. Rat-liver bilirubin glucuronyltransferase activity increased only about 50% by addition of 2 mM UDP-*N*-acetylglucosamine, while 2.9-fold activation was achieved with Triton X-100. 2 mM *N*-ethylmaleimide abolished the effect of UDP-*N*-acetylglucosamine, but not that of Triton X-100 as also found for the other acceptors.

DISCUSSION

The present results indicate that the active site of glucuronyltransferase is not dependent on thiol-groups, in contrast to an earlier study on the effect of sulfhydryl-reacting agents on this enzyme[11]. However, these apparent contradictory results are explained by the different kinetics of "native" and sulfhydryl-blocked enzyme (Fig. 4). STOREY[11] in his study used 0.098 mM UDP-glucuronate in the experiments with *p*-nitrophenol as acceptor. At such low substrate concentration complete inhibition was found also in the present study.

Since ample evidence suggests the presence of multiple glucuronyltransferases[13–15], five different acceptors were used in the present study. In principle the same effects of the sulfhydryl-reacting agents were found with all acceptors. However, bilirubin glucuronyltransferase from mouse-liver was not activated by UDP-*N*-acetylglucosamine in agreement with earlier results[2].

Since UDP-*N*-acetylglucosamine probably is no cofactor for glucuronide synthesis (non-additive activation of *p*-nitrophenol glucuronyltransferase by UDP-*N*-acetylglucosamine and Triton X-100), and since it is a metabolite present in liver cells[16], it was postulated in an earlier report[2] that UDP-*N*-acetylglucosamine might be an allosteric activator. The term allosteric is used here in its broadest sence, implicating a binding site for the activator separate from the active site, and an indirect effect of the ligand favouring a special configuration of the enzyme with high activity[17].

One of the most characteristic properties of allosteric enzymes is the phenomenon called desensitization which implies that different kinds of treatment (dialysis, heating, treatment with thiol reagents *etc.*) which do not destroy the catalytic properties of an enzyme, makes it insensitive towards the allosteric effector[17].

The desensitization of glucuronyltransferase towards UDP-*N*-acetylglucosamine by sulfhydryl-reacting agents therefore supports the hypothesis that the nucleotide is an allosteric activator. The fact that unspecific agents like Triton X-100, digitonin and deoxycholate are capable of promoting a similar activation[2] is not necessarily contradictory to this suggestion.

The pH optimum of both nucleotide and detergent activated *p*-nitrophenol glucuronyltransferase is at 6.2–6.6, which is different from that of the spontaneously activated enzyme[2], which exhibits maximal activity at pH 7.6–7.8. This probably reflects that a similar conformational change of the enzyme is promoted by UDP-*N*-acetylglucosamine and detergents.

The kinetics of "native" glucuronyltransferase do not correspond to the typical findings for allosteric enzymes which exhibit sigmoidal substrate saturation curves[18]. The thiol-blocked glucuronyltransferase exhibited, however, some similarity in this respect indicating a homologous allosteric effect[18] of UDP-glucuronate.

Since glucuronyltransferase is membrane-bound one cannot answer the question whether the UDP-*N*-acetylglucosamine binding-site is located on the enzyme molecule itself or on an adjacent microsomal membrane protein (or lipid) capable of affecting the catalytic activity of the enzyme. The fact that mouse-liver bilirubin glucuronyltransferase is not activated by UDP-*N*-acetylglucosamine whereas Triton X-100 has good effect favours the suggestion that UDP-*N*-acetylglucosamine binds to the enzyme itself (bilirubin may be conjugated by a separate enzyme). A possible general effect of UDP-*N*-acetylglucosamine on microsomal membrane permeability is also contradicted by the fact that microsomal glucose-6-phosphatase, which in agreement with earlier results[10] was activated by Triton X-100, was unaffected by 1, 2 and 4 mM UDP-*N*-acetylglucosamine (A. WINSNES, unpublished observations).

Glucuronyltransferase is intimately associated with microsomal membranes and has also been shown to be dependent on phospholipid micelles for catalysis[19]. Although the enzyme can be partly "solubilized" by detergents and ultrasonic oscillation as defined by non-sedimentation upon high-speed centrifugation[1–3,20,21], electron micrographs of the partly purified fraction obtained revealed intact membraneous structures[21].

The "solubilized" or partly purified glucuronyltransferase preparations hitherto obtained were not activated by UDP-*N*-acetylglucosamine[1,3]. This fact has been interpreted on the basis that the effect of UDP-*N*-acetylglucosamine is solely competitive inhibition of the pyrophosphatase breakdown of UDP-glucuronate, since no pyrophosphatase activity was found in the solubilized fraction[3]. Several data contradict this interpretation: The activating effect of UDP-*N*-acetylglucosamine increases in the UDP-glucuronate concentration range 0.25–8.0 mM (ref. 2) which is opposite to the expected if pyrophosphatase inhibition is the explanation. In neonatal rats UDP-*N*-acetylglucosamine activates *o*-aminophenol glucuronyltransferase three times as effectively as in adult female rats[22], although the pyrophosphatase activity is rather low at birth compared with adult age in rats[23]. Furthermore, the presence of 10 mM EDTA (which completely inhibits the UDP-glucuronate pyrophosphatase[24]) in the incubation mixtures neither affected "native" nor UDP-*N*-acetylglucosamine activated mouse-liver *p*-nitrophenol glucuronyltransferase. (A. WINSNES, unpublished observations). In conclusion, though UDP-*N*-acetylglucos-

amine inhibits UDP-glucuronate pyrophosphatase this effect seems to be of minor importance in explaining the activation of mouse-liver glucuronyltransferase observed here.

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