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INHIBITION OF HEPATIC UDPGLUCURONYLTRANSFERASE BY NUCLEOTIDES

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SUMMARY

1. Various purine and pyrimidine nucleotides were tested for their effect *in vitro* on different forms of mouse-liver *p*-nitrophenol and *o*-aminophenol UDPglucuronyltransferase (UDPglucuronate glucuronyltransferase (acceptor unspecific), EC 2.4.1.17).

2. "Native" glucuronyltransferase was slightly inhibited by both UMP, UDP, UTP and CMP. The nature of this inhibition was not further evaluated.

3. UDP-*N*-acetylglucosamine-activated glucuronyltransferase was inhibited competitively with respect to the activator by UMP and to a lesser degree by UDP with *p*-nitrophenol as acceptor and also slightly by UTP when *o*-aminophenol was acceptor.

4. Detergent-activated glucuronyltransferase exhibited an inhibition pattern opposite to that found for UDP-*N*-acetylglucosamine-activated enzyme. With *p*-nitrophenol as acceptor UDP and UTP inhibited the enzyme strongly due mainly to competition with UDPglucuronate, whereas UMP did not inhibit. When *o*-aminophenol served as acceptor the slight inhibition by UDP and UTP was non-competitive with respect to UDPglucuronate. *p*-Nitrophenol glucuronyltransferase activated by ageing, sonication, alkaline pH, *p*-chloromercuribenzoate and phospholipase C (EC 3.1.4.3) exhibited an inhibition pattern similar to the detergent-activated enzyme.

5. The UDP-*N*-acetylglucosamine-activated *p*-nitrophenol glucuronyltransferase would be little affected by the concentrations of the inhibiting nucleotides encountered in the cell, whereas the detergent-activated form of enzyme (if it exists in the cell) would be strongly inhibited by UDP and UTP in concentrations expected in the cell. The activated forms of *o*-aminophenol glucuronyltransferase would be little affected by the levels of inhibiting nucleotides found *in vivo*.

INTRODUCTION

Hepatic UDPglucuronyltransferase (UDPglucuronate glucuronyltransferase (acceptor unspecific), EC 2.4.1.17) can be strongly activated at V by a variety of

agents and treatments (detergents[1–7], ageing[1,2,5], sonication[6,8], alkaline buffers[6,9,10], phospholipases[6,11,12] and sulfhydryl-blocking agents[13,14]) most of which are rather unspecific and "unphysiological". There are mainly two possible explanations of this enzyme activation: (1) Most of the available enzyme protein might be located on the inner side of the microsomal membranes which might constitute a permeability barrier towards the substrate(s). Charged substances of molecular weight above 90 have been shown to penetrate with difficulty[15]. UDPglucuronic acid which is synthesized in the cytosol[16] would therefore have to be actively transported to the inside of the microsomal vesicles. (2) Alternatively there might be both active and inactive forms of the enzyme protein itself; the activators promoting conversion to the active form. Some authors have favoured the first[3,4] and some the second[6,13] model.

It is tempting to suggest that the activation observed *in vitro* might reflect regulatory mechanisms of importance in the intact cell though direct evidence for such regulation is lacking. If indeed glucuronyltransferase is regulated one would expect to find compounds acting more specifically on this enzyme at concentrations which might be encountered in the cell. UDP-*N*-acetylglucosamine is such a compound. It does not affect enzyme already activated by detergents, ageing or phospholipase C (EC 3.1.4.3), but has a strong activating effect on "native" enzyme[2,12].

Striking differences between UDP-*N*-acetylglucosamine-activated and detergent-activated *p*-nitrophenol glucuronyltransferase with respect to inhibition by UMP, UDP and UTP are demonstrated in the present contribution. Enzyme activated by ageing, sonication, alkaline pH, *p*-chloromercuribenzoate and phospholipase C exhibited inhibition patterns similar to that of detergent-activated enzyme. Evidence is presented that the UDP-*N*-acetylglucosamine-activated enzyme can be slightly inhibited by UMP and UDP which seem to compete with the activator, whereas strong inhibition by UDP and UTP, caused by competition with UDP-glucuronate, is characteristic for detergent-activated *p*-nitrophenol glucuronyltransferase. *o*-Aminophenol glucuronyltransferase was much less sensitive towards the inhibitors, a fact which would accord with the suggestion that different enzymes are involved in the conjugation of *p*-nitrophenol and *o*-aminophenol[18,19].

MATERIALS AND METHODS

The animals used were female NMRI/BOM mice of 20–30 g weight. The post-mitochondrial fraction of liver homogenate was assayed for *p*-nitrophenol and *o*-aminophenol glucuronyltransferase activity as described elsewhere[2].

RESULTS

Effects of nucleotides on "native" glucuronyltransferase

Both UMP, UDP and UTP in concentrations of 2 mM reduced the "native" *o*-aminophenol glucuronyltransferase activity to about 50% though there was no further inhibition by increasing the inhibitor concentration to 4 mM (Fig. 1). When *p*-nitrophenol served as acceptor the nucleotides were less potent inhibitors; 2 mM UTP and UDP reduced the activity to 70–80% whereas UMP had only a very slight effect (Fig. 1). The nature of this inhibition was not evaluated further since kinetic

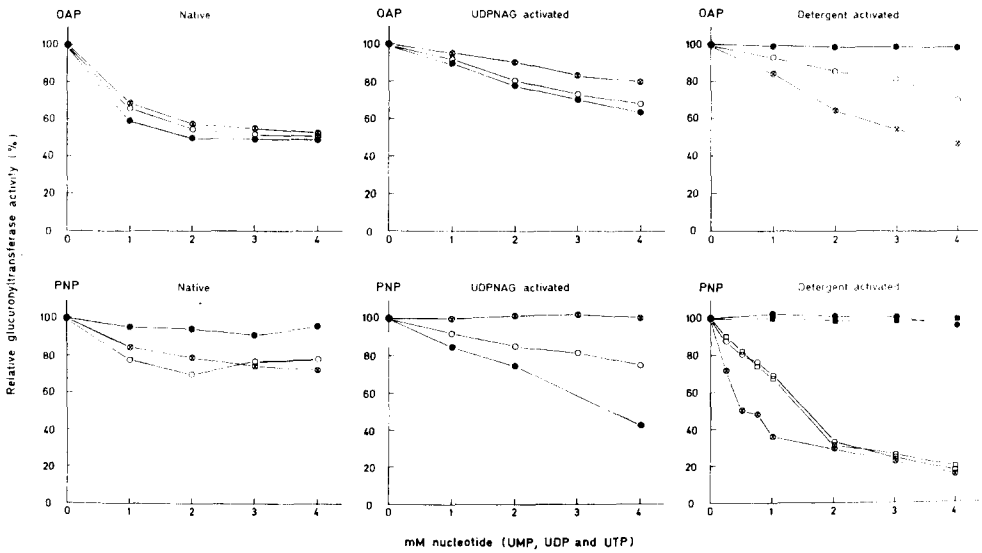

Fig. 1. Effects of uridine nucleotides on different forms of glucuronyltransferase. Glucuronyltransferase activity was assayed in mouse liver suspension with 2 mM UDPglucuronate and either *o*-aminophenol (OAP) or *p*-nitrophenol (PNP) as acceptor. The concentrations of UMP, UDP and UTP were varied between 0 and 4 mM. Filled symbols represent enzyme with UMP added, open symbols represent enzyme with UDP and ⊗ represents assays where UTP was added. Non-activated enzyme (native) as well as enzyme activated by UDP-*N*-acetylglucosamine (UDPNAG), and detergents were tested. Digitonin (0.2%) was used as activator when *o*-aminophenol was acceptor and both digitonin (□, ■) and 0.05% Triton X-100 (○, ●) was used with *p*-nitrophenol as acceptor. The enzyme activities were calculated relative to that obtained without inhibitor present.

studies of "native" glucuronyltransferase are very difficult since the low activity results in poor accuracy.

Effects of nucleotides on UDP-N-acetylglucosamine-activated glucuronyltransferase

All three uridine nucleotides inhibited slightly when *o*-aminophenol was acceptor whereas UTP had no effect on *p*-nitrophenol glucuronyltransferase though UDP and especially UMP were clearly inhibitory.

If there are two nucleotide-binding sites on glucuronyltransferase, one for binding of UDPglucuronate (active site) and one for binding of UDP-*N*-acetylglucosamine (effector site) analogous nucleotides might possibly bind to either site. Fig. 3 illustrates that UMP and UDP inhibit the UDP-*N*-acetylglucosamine-activated *p*-nitrophenol glucuronyltransferase competitively with respect to the activator. With *o*-aminophenol similar results were obtained at the higher concentrations of UDP-*N*-acetylglucosamine. Anomalous kinetics were, however, observed in the lower activator concentration range with UMP and UDP as inhibitors as was observed also with 4 mM UMP in the case of *p*-nitrophenol glucuronyltransferase. This might be partly explained by the possible presence of at least two types of enzyme, both "native" and UDP-*N*-acetylglucosamine-activated. The first type probably predominates at low concentrations of UDP-*N*-acetylglucosamine, while the latter predominates at higher concentrations of activator.

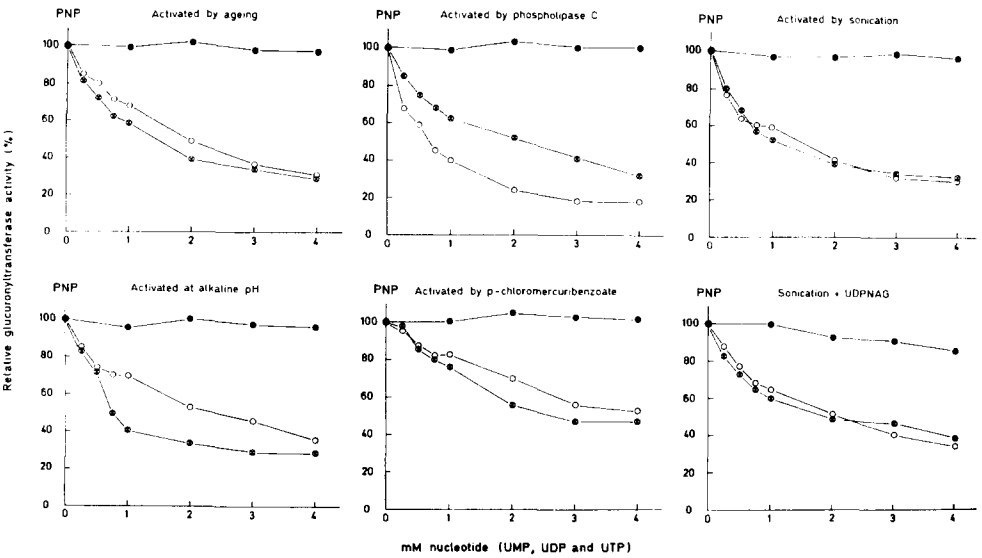

Fig. 2. Effects of uridine nucleotides on different forms of *p*-nitrophenol glucuronyltransferase. Assay conditions and symbols are as described for Fig. 1. The enzyme suspension activated by ageing was obtained by storage at 20 °C for 24 h. Phospholipase-treated enzyme was obtained by incubation of a liver suspension with phospholipase C (from *Cl. perfringens*, Koch-Light laboratories) at an approximate liver protein to phospholipase protein ratio of 10:1 for 15 min at 20 °C with 2.7 mM $CaCl_2$ and 12 mM Tris–maleate buffer, pH 8.0. The reaction was stopped by addition of EDTA to a final concentration of 4.4 mM. A 5-fold dilution followed when the glucuronyltransferase assay was performed at pH 7.4. Liver suspension was activated by sonication for 10 s repeated six times as described by Henderson[8]. This sonicated suspension was assayed both without and with 2 mM UDP-*N*-acetylglucosamine (UDPNAG) added in the incubation mixture. Another enzyme preparation was pretreated with glycine–NaOH buffer, pH 10.3, for 30 min at 4 °C before assay. The enzyme suspension was also pretreated with 3.3 mM. *p*-chloromercuribenzoate for 30 min before the enzyme assay where the *p*-chloromercuribenzoate concentration was 1 mM. PNP, *p*-nitrophenol.

Other activator analogues, UDP-glucose and *N*-acetylglucosamine in 2 mM concentration exhibited no effect on UDP-*N*-acetylglucosamine-activated *p*-nitrophenol glucuronyltransferase.

Effects of uridine nucleotides on detergent-activated glucuronyltransferase

In contrast to the findings with UDP-*N*-acetylglucosamine-activated enzyme, UMP had no inhibiting effect when Triton X-100 or digitonin were used as activators (Fig. 1). On the other hand, UDP and especially UTP inhibited strongly when *p*-nitrophenol was acceptor whereas the effect was comparatively much smaller with *o*-aminophenol as substrate.

In the case of *p*-nitrophenol glucuronyltransferase there was an initial phase of rapidly declining activity whereas at higher concentrations of inhibitor the activity declined more slowly (Fig. 1). If only one type of enzyme was present one would expect to find a hyperbolic inhibition curve approaching zero activity asymptotically. The negative cooperativity with respect to UDPglucuronate of *p*-nitrophenol glucuronyltransferase described elsewhere[12] suggested the possible presence of two different forms of detergent-activated *p*-nitrophenol glucuronyltransferase in mouse liver. This suggestion is strengthened by the atypical inhibition curves shown in Figs 1 and 2

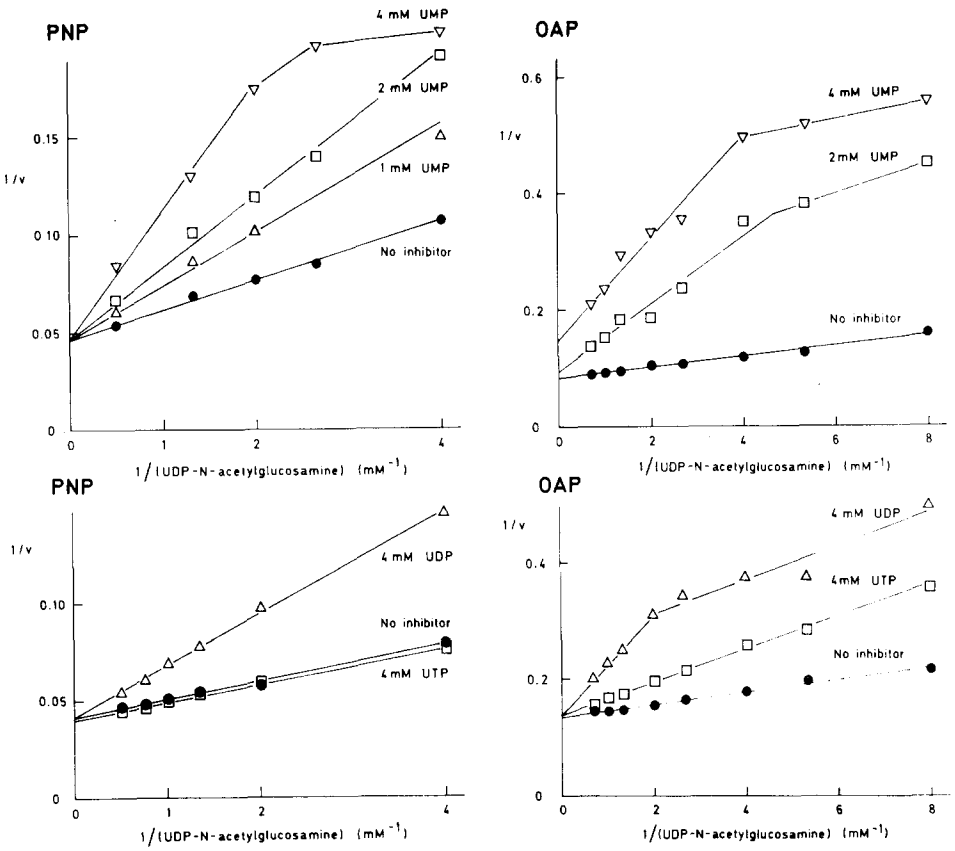

Fig. 3. Inhibition by nucleotides of UDP-*N*-acetylglucosamine-activated glucuronyltransferase. Mouse liver suspension was assayed for glucuronyltransferase activity with 2 mM UDPglucuronate and either 0.5 mM *p*-nitrophenol (PNP) or *o*-aminophenol (OAP) as acceptors. The UDP-*N*-acetylglucosamine concentrations were varied between 0.125 mM (OAP) or 0.25 mM (PNP) to 2.0 mM and additions of UMP, UDP and UTP were as indicated. The enzyme activities were calculated as μmoles/g wet weight liver per h.

indicating that one type of enzyme is highly sensitive towards the inhibitors whereas the other type is not.

The inhibition by UDP and UTP was analysed in Lineweaver–Burk plots with respect to varying UDPglucuronate concentrations. With *o*-aminophenol as acceptor the inhibition by UDP and UTP was of the non-competitive type (only the experiment with UDP is shown in Fig. 4A). When *p*-nitrophenol was acceptor a competitive type of inhibition was found with UDP whereas UTP exhibited a mixed type of inhibition (Fig. 4B).

Both "solubilized" and non-solubilized enzyme were competitively inhibited by UDP (Figs 4C and 4D). The inhibition was strongest in the case of non-solubilized enzyme (Fig. 4C). At 2 mM UDPglucuronate the activity in the high speed supernatant was reduced to 66% by 1 mM of UDP whereas the reduction was to 35% in the pellet resuspended to contain an equivalent amount of Triton X-100. When the high speed pellet was washed twice to remove Triton X-100 the inhibition was even

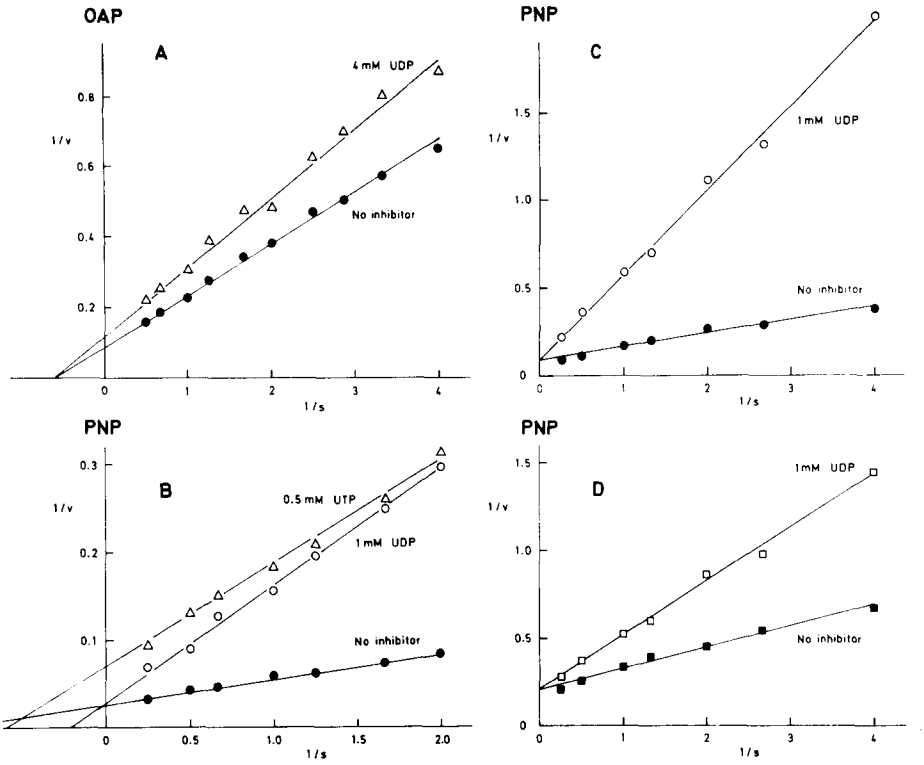

Fig. 4. Inhibition by nucleotides of detergent-activated glucuronyltransferase. Glucuronyltransferase activities were assayed with either 0.5 mM *o*-aminophenol (OAP) or *p*-nitrophenol (PNP) as acceptor at UDPglucuronate concentrations varying between 0.25 and 4.0 mM. Additions of UDP and UTP are as indicated. The enzyme activities (v) were calculated as μmoles/g wet weight liver per h. The curves in A represent enzyme suspension assayed with 0.2% (w/v) digitonin present. The curves in B represent enzyme suspension assayed with 0.05% (w/v) Triton X-100 present. The curves in C represent the resuspended pellet obtained by centrifugation at 40 000 $\times$ g for 90 min after treatment of microsomes with 0.25% (w/v) Triton X-100 for 1 h at 0 °C, whereas the curves in D represent the corresponding high speed supernatant with "solubilized" enzyme.

more pronounced, with only 10% of the activity remaining at the same concentrations of substrate and inhibitor as given above. Thus treatment of the enzyme with Triton X-100 results in an irreversible change of the enzyme to a type highly susceptible to inhibition by UDP.

In Figs 4A, 4B and 4C the non-linearity of the double-reciprocal plots described earlier for *p*-nitrophenol glucuronyltransferase[12], are masked. By choosing another ordinate scale the abrupt transition of the kinetic curve (indicating negative cooperativity) for the non-inhibited enzyme becomes clearly visible. No abrubt transition was found, however, in the case of nucleotide-inhibited enzyme. The inhibition by UDP was therefore competitive with respect to the mean curve for the postulated two forms of enzyme.

Effects of uridine nucleotides on p-nitrophenol glucuronyltransferase activated by ageing, phospholipase C, sonication, alkaline pH and p-chloromercuribenzoate

Fig. 2 shows that a row of different activators resulted in appearance of an

inhibition pattern similar to that of detergent-activated enzyme. *p*-Chloromercuribenzoate is the least effective activator[13] resulting in only about a 2-fold increase in enzyme activity at the substrate concentrations used, whereas phospholipase C activates 4-fold[12] and the other activation principles enhance the activity 6–7-fold[2,6,8]. UTP was slightly more effective as inhibitor with all preparations except that treated with phospholipase C where UDP was the most potent inhibitor. Most inhibition curves were hyperbolic, though inhibition levelled off at activities well above zero again giving the impression that another less sensitive form of enzyme was present.

The sonicated enzyme preparation was also assayed with 2 mM UDP-*N*-acetylglucosamine present in the incubation mixture. This did not reverse the inhibition type to that characteristic of UDP-*N*-acetylglucosamine-activated enzyme. A similar experiment was performed in which the sonicated enzyme suspension was preincubated with 10 mM UDP-*N*-acetylglucosamine for 15 min at 37 °C. In this case also there was no reversion of the inhibition pattern towards that seen for UDP-*N*-acetylglucosamine-activated enzyme. This would indicate either that the sonicated enzyme was desensitized towards UDP-*N*-acetylglucosamine so that no conformational change could take place, or alternatively, that UDP-*N*-acetylglucosamine only exerts its effect in presence of "native" microsomal vesicles as found in fresh homogenates prepared by gentle homogenization.

Effects of other substrate and activator analogues of glucuronyltransferase

"Native", UDP-*N*-acetylglucosamine and Triton X-100-activated *p*-nitrophenol glucuronyltransferase was assayed in presence of 2 mM of the following compounds: UDPglucose, CMP, CTP, TMP, TTP, AMP, ATP, GMP, IMP, $NADP^+$ and *N*-acetylglucosamine. Most experiments were repeated 4 times. Effects of less than 10% inhibition or activation were considered insignificant. 2 mM CMP lowered "native" and UDP-*N*-acetylglucosamine-activated enzyme activity to 70 and 80% of the control, whereas TMP inhibited to the same degree only the latter enzyme preparation. ATP increased the activity of UDP-*N*-acetylglucosamine-activated enzyme by about 30%. None of the compounds listed had any significant effect on detergent-activated glucuronyltransferase. These results indicate that the inhibition of glucuronyltransferase by nucleotides was rather specific and confined to the pyrimidine derivatives.

DISCUSSION

The possibility of product inhibition of glucuronyltransferase has attracted little interest until Hänninen and Marniemi[20] recently showed that glucuronides inhibited the enzyme by competition with UDPglucuronate. Götze *et al.*[21] reported that their detergent-solubilized steroid glucuronyltransferase from the small intestine of pig was strongly inhibited by UDP. The present report shows that not only UDP, but also UTP and UMP may inhibit glucuronyltransferase, albeit by different mechanisms. The main purpose of this contribution is, however, to compare the inhibition characteristics of different forms of activated glucuronyltransferases. Clear differences with respect to inhibition by nucleotides between the UDP-*N*-acetylglucosamine-activated form of glucuronyltransferase and that obtained by treatment with more unspecificly acting agents like detergents were demonstrated.

It is still open to question whether UDP-*N*-acetylglucosamine exerts its effect primarily on glucuronyltransferase itself or on the microsomal membrane. If the effect is that of promoting conversion of inactive glucuronyltransferase molecules to an active form, the latter must have another conformation than that obtained by detergent activation since the inhibition patterns are so clearly different (see Fig. 1). If, on the other hand, UDP-*N*-acetylglucosamine acts on the microsomal membrane, one would have to postulate that it increased the permeability of UDPglucuronate alone, such that UDP and UTP did not gain access to the majority of the active sites hence giving no or only slight inhibition. Detergents, sonication *etc.* (see Figs 1 and 2) probably destroy the permeability barrier of microsomal membranes towards any compound, thereby revealing the strong inhibition by UDP and UTP.

Whether the nucleotides inhibiting glucuronyltransferase *in vitro* have any function as physiological regulators of glucuronide synthesis in the cell is open to speculation. This question is especially difficult since it remains unknown to what extent the different forms of glucuronyltransferase exist in the cell.

The concentrations (μmoles per g wet tissue) in rat liver of the most important nucleotides with effect on glucuronyltransferase activity *in vitro* were found by Keppler *et al.*[22] to be as follows: UDP-*N*-acetylhexosamine (both glucose and galactose derivatives) 0.32; UDPglucuronate 0.28; UTP + UDP 0.34; and UMP 0.04.

The apparent K_a value with respect to UDP-*N*-acetylglucosamine (with *p*-nitrophenol as acceptor) was 0.41 mM (0.33, 0.44 and 0.46 mM in three separate experiments) which is only slightly higher than the concentration of the activator found in rat liver. Therefore, if the concentrations are similar in mouse liver, one would expect a significant proportion of the glucuronyltransferase to be of the UDP-*N*-acetylglucosamine-activated type which is not inhibited by UTP and only weakly inhibited by UDP and UMP.

If, however, a significant proportion of the enzyme was present in a form similar to the detergent-activated type this would be highly susceptible to inhibition by the levels of UDP and UTP found in the cell, especially since the concentration of UDPglucuronate is of the same order of magnitude as the inhibitors. These considerations are of course valid only if there is no compartmentation of the nucleotides in question.

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