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# STUDIES ON THE ACTIVATION *IN VITRO* OF GLUCURONYLTRANSFERASE

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SUMMARY

1. The relationship between the different activating principles acting on glucuronyltransferase (UDP-glucuronate glucuronyltransferase, EC 2.4.1.17) *in vitro* has been studied using five acceptor substrates (bilirubin, *o*-aminophenol, 4-methylumbelliferone, *p*-nitrophenol and phenolphthalein) in assay of enzyme activity.

2. Preincubation of mouse and rat-liver suspensions *in vitro* resulted in an increased activity of glucuronyltransferase which was highly variable depending on the acceptor substrate used. While 4-methylumbelliferone glucuronyltransferase exhibited an activity that was 45 times the initial one at maximum, the *o*-aminophenol enzyme was not activated by this procedure.

3. Detergents (Triton X-100 and digitonin) and UDP-*N*-acetylglucosamine were also found to activate glucuronyltransferase; only bilirubin glucuronyltransferase was not activated by UDP-*N*-acetylglucosamine.

4. Activation of glucuronyltransferase could not be increased by combining preincubation, detergents or UDP-*N*-acetylglucosamine, whereas activation of rat-liver *o*-aminophenol glucuronyltransferase by detergents and UDP-*N*-acetylglucosamine was strongly potentiated by diethylnitrosamine.

5. The slightly different kinetics of nonactivated and activated enzyme that were found at varying substrate (UDP-glucuronic acid, *p*-nitrophenol) concentrations could not explain the activation observed.

6. The results are compatible with an activation by preincubation, detergents and UDP-*N*-acetylglucosamine due to exposition of active sites of glucuronyltransferases that have been nonfunctioning in unactivated tissue homogenates.

7. The degree of activation at constant detergent concentration was the same over a wide range of enzyme-protein concentrations.

8. The pH optimum for detergent-activated *p*-nitrophenol glucuronyltransferase was found at 6.2–6.6, while the activity towards bilirubin and *o*-aminophenol was maximum at pH 7.6. Detergent-activated 4-methylumbelliferone and phenolphthalein glucuronyltransferases, on the other hand, revealed about the same activity in the range between pH 6.2 and 7.6.

9. The inhibition of glucuronyltransferase at higher concentrations of detergents was shown to be almost completely reversible upon dilution.

10. About 40% of the glucuronyltransferase activity was recovered in the high-speed supernatant when rat- and mouse-liver suspensions were treated with Triton X-100. The recovery was about 110% in the pellet and supernatant together. No separation of glucuronyltransferases was achieved by solubilization.

## INTRODUCTION

Liver microsomal glucuronyltransferase (UDP-glucuronate glucuronyltransferase, EC 2.4.1.17) has been activated *in vitro* by various means[1–8]. Activation of *p*-nitrophenol glucuronyltransferase from rat and guinea pig liver by UDP-*N*-acetylglucosamine and ATP was reported by POGELL AND LELOIR[1], while ISSELBACHER *et al.*[2] and TOMLINSON AND YAFFE[3] reported activation of the corresponding enzyme when rabbit-liver microsomes were treated with snake venom. When rabbit-liver microsomes were dialyzed overnight at pH 9.0, 10–20-fold increased activity of phenolphthalein glucuronyltransferase was observed[4]. Both spontaneous and detergent activation of *p*-nitrophenol glucuronyltransferase was described by LUEDERS AND KUFF[5], and the effect of deoxycholate and digitonin on bilirubin glucuronyltransferase has recently been reported by VAN ROY AND HEIRWEGH[6] and HEIRWEGH AND MEUWISSEN[7]. The carcinogen diethylnitrosamine has been shown to activate *o*-aminophenol and paracetamol glucuronyltransferases in rat liver, while no activation was found with *p*-nitrophenol, phenolphthalein or menthol as substrates[8].

However, little or no attention has been paid to the relationship, if any, between the different activating principles acting on glucuronyltransferase. In the present paper, special notice is paid to this item.

Since there is ample evidence that several glucuronyltransferases exist[2,3,9–11], it was also thought to be of interest to study the activation *in vitro* of glucuronyltransferase measured with several of the acceptor substrates in common use. Although all the enzymes tested (bilirubin, *o*-aminophenol, 4-methylumbelliferone, *p*-nitrophenol, and phenolphthalein glucuronyltransferases) were activated by one or the other method, interesting differences were found.

Activation *in vitro* obviously offers the possibility of making the assay methods more sensitive. This may be of special value when assaying glucuronyltransferase activity in tissues with low activity or when limited amounts of tissue (*e.g.*, needle biopsy material) are available. The present report evaluates some questions of importance in connection with routine use of detergents as activators *in vitro* of glucuronyltransferase. The optimal concentrations of detergents and UDP-glucuronic acid, the effect of varying enzyme-protein concentrations, as well as the pH optimum for detergent-activated enzyme were determined.

The present report also describes a new method for solubilizing glucuronyltransferase using Triton X-100.

## MATERIALS AND METHODS

### *Chemicals*

Chemicals of purest grade were obtained from the following sources: UDP-

glucuronide sodium salt from Boehringer; UDP-*N*-acetylglucosamine sodium salt, ATP sodium salt, bovine albumin, Triton X-100, deoxycholic acid, digitonin, and bovine-liver β-glucuronidase from Sigma Chemical Co.; bilirubin, *p*-nitrophenol, 4-methylumbelliferone, phenolphthalein-β-D-glucuronide, and *o*-aminophenylglucuronide from Koch-Light Laboratories; *o*-aminophenol, 2-pentanon, and butyl acetate from Fluka; saccharo-1,4-lactone from Calbiochem; ammonium sulphamate from Riedel-de-Haën; *N*-1-naphthylethylenediamine·2 HCl from Eastman Organic Chemicals; diethylnitrosamine from Schuchardt.

*Animals*

Animals were female NMRI/Bom mice (20–30 g) and female Wistar rats (150–250 g), all fed *ad libitum*.

*Preparation of enzyme suspensions*

Rats were killed by decapitation and mice by dislocation of the vertebrae. The liver and kidneys were removed at once, weighed and cooled on ice. The tissues were then cut with scissors prior to grinding in a glass homogenizer with Teflon pestle. Grinding was done for 1–2 min with pestle speed at 1000 rev./min with the homogenizer submerged in ice water. Ice-cold isotone KCl solution was used as suspending medium with 2 g of tissue per 10 ml of homogenate (20%). Supernatant I (SI) was prepared by spinning the homogenate at 2000 × *g* for 15 min. All handling of enzyme suspensions was done at 0–4°, and assays were done within 3–4 h after killing of the animal, unless stated otherwise.

*Assay of glucuronyltransferase*

Glucuronyltransferase assays were performed with the following acceptor substrates: bilirubin[6], *o*-aminophenol[12,13], 4-methylumbelliferone[14], *p*-nitrophenol[2,15], and phenolphthalein[16]. For purpose of standardization, the methods described in refs. 2, 6, 12–16 were modified in the following respects. The final concentration of acceptor substrates was 0.5 mM and of UDP-glucuronic acid 2.0 mM. The buffer was Tris–maleate (pH 7.4) in a final concentration of 75 mM. In the bilirubin, *o*-aminophenol and 4-methylumbelliferone incubation solutions, 10 mM $MgCl_2$ was included. Bovine albumin, 20 mg/ml, was used in the bilirubin, 4-methylumbelliferone and phenolphthalein assay systems to keep the acceptor substrates in solution. 96% ethanol was used to dissolve *p*-nitrophenol, and the incubation solution therefore contained 0.05% ethanol. EDTA was used to stabilize alkaline bilirubin[6], and ascorbic acid was added to protect *o*-aminophenol against oxidation[12,13], thus giving final concentrations of 0.8 mM EDTA and 1.0 mM ascorbic acid in the respective assay systems. Incubation solutions (final volume 0.5 ml) were kept in stoppered glass tubes (1 cm × 12 cm) and incubated aerobically for 30 min at 37° in a shaking water bath (40 strokes per min). When assaying nonactivated liver glucuronyltransferase for activity towards bilirubin and *o*-aminophenol, enzyme corresponding to 40 mg wet wt. of tissue were used, while 20 mg were used in the case of *p*-nitrophenol, 4-methylumbelliferone and phenolphthalein. When assaying fully activated liver glucuronyltransferase for activity towards bilirubin and *o*-aminophenol, enzyme corresponding to 20 mg wet wt. of tissue was used, for *p*-nitrophenol and phenolphthalein 10 mg and for 4-methylumbelliferone 2.5 mg were used. Incubations usually were in duplicate. Blanks containing all reagents

except UDP-glucuronic acid were kept at 0°, and stopping reagents were added immediately after addition of enzyme. Stopping reagents were glycine buffer (pH 2.6) for bilirubin, 0.5 mM trichloroacetic acid for *p*-nitrophenol and *o*-aminophenol, glycine buffer (pH 10.4) for 4-methylumbelliferone and 96% ethanol for the phenolphthalein system. Bilirubin and *o*-aminophenyl glucuronides were estimated after diazotization at pH 2.6 and 2.4, respectively, as described in refs. 6 and 12. In routine assays, *p*-nitrophenyl (pH 9.0) and phenolphthalein glucuronides (pH 10.4) were estimated by reduction in absorbance at 400 and 550 nm, respectively, while 4-methylumbelliferyl glucuronide (pH 10.4) was estimated by reduction in fluorescence at 460 nm (activation energy at 375 nm).

*Hydrolyzing glucuronides with β-glucuronidase*

The methods based on reduction in absorbance and fluorescence were checked by hydrolyzing the glucuronides formed. pH was adjusted to 5.0, and 2000 Fishman units of bovine liver β-glucuronidase were added before incubation for 30 min at 37°. The results were compared with controls without β-glucuronidase and controls with both β-glucuronidase and saccharo-1,4-lactone (5 mM) added.

*Assay of β-glucuronidase*

β-Glucuronidase was assayed with a method slightly modified after TALALAY *et al.*[17]. 1 mM phenolphthalein glucuronide, 75 mM acetate buffer (pH 4.5), 0.2% Triton X-100 and enzyme corresponding to 20 mg wet wt. of tissue in a final volume of 0.5 ml were incubated at 37° for 30 min. Free phenolphthalein (pH 10.4) was estimated at 550 nm.

*Preincubation of enzyme*

Preincubation of enzyme was done with enzyme suspensions (SI) and intact tissue kept aerobically in stoppered glass tubes in ice or in a water bath at 37°. In the case of intact tissue, 0.154 M KCl solution was added before preincubation, and homogenization of the preincubated mixture was done before assay of enzyme activity. Centrifugation of this homogenate was omitted since a considerable amount of the glucuronyltransferase activity was lost in the $2000 \times g$ pellet.

## RESULTS

*Preincubation of enzyme*

A spontaneous activation of glucuronyltransferase was observed when rat-liver suspension (SI) was kept at 37° (Fig. 1). In an initial lag period, the activity of glucuronyltransferase was either quite constant or decreasing. Activity towards all acceptor substrates then increased, but to very different degrees. While the *o*-aminophenol glucuronyltransferase activity remained below the initial one throughout the experiment, the 4-methylumbelliferone glucuronyltransferase exhibited a maximal activity 45 times the initial one after 9 h at 37°. Both β-glucuronidase activity in suspension (SI) kept at 37° and *p*-nitrophenol glucuronyltransferase activity in suspension (SI) kept at 0° remained essentially unchanged during the experiment. Similar results were obtained during preincubation of mouse-liver suspension (SI) both at 37 and 0°, although the lag period was about as many days in the 0° experiments as it was hours

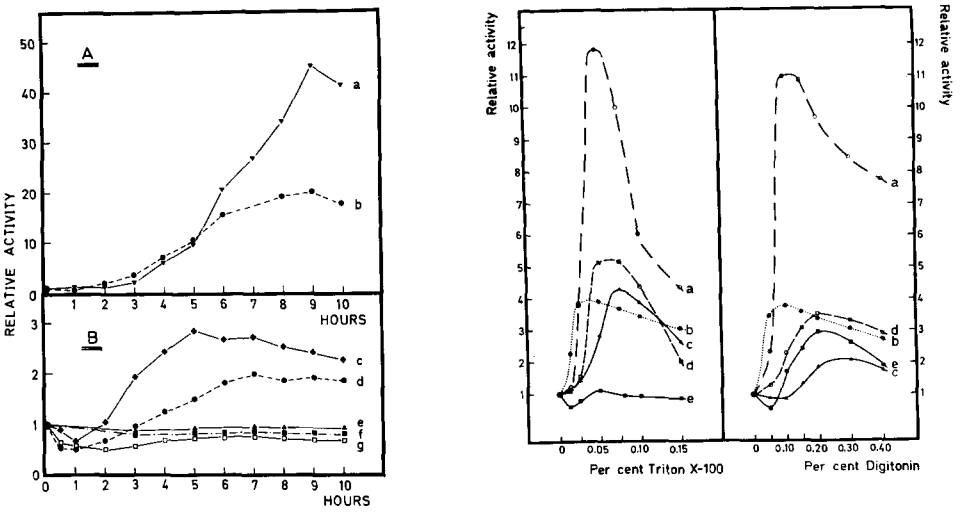


Fig. 1. Glucuronyltransferase activity in rat-liver suspension during preincubation at 37°. A and B. 10% rat-liver suspension (SI) was preincubated (without additions) at 37° and assayed at regular intervals for 4-methylumbelliferone (a), phenolphthalein (b), *p*-nitrophenol (c), bilirubin (d), and *o*-aminophenol (g) glucuronyltransferase activity, and *β*-glucuronidase activity (e). Some of the enzyme suspension was kept at 0° during the experiment and assayed for *p*-nitrophenol glucuronyltransferase activity (f). The enzyme activities are given relative to the initial one. The figure represents one typical experiment.

Fig. 2. The effect of Triton X-100 and digitonin on mouse-liver glucuronyltransferase. Mouse-liver suspension (SI) was assayed for glucuronyltransferase activity with different concentrations of Triton X-100 and digitonin added in the test vials. Acceptor substrates were: 4-methylumbelliferone (a), *p*-nitrophenol (b), bilirubin (c), phenolphthalein (d), and *o*-aminophenol (e). Activities are given relative to the one without detergent added. The curves represent the mean of two experiments.

in the 37° experiments. Spontaneous activation of *p*-nitrophenol glucuronyltransferase was also observed in kidney suspension (SI) and intact liver during preincubation at 37°. The length of the lag periods and the degree of activation varied to some extent from experiment to experiment as did the length of time before coagulation of the enzyme suspension took place. (Coagulation resulted in loss of all glucuronyltransferase activity.) The same pattern was, however, always found, showing an independent course of the activity towards each of the five acceptor substrates.

*Detergent activation of glucuronyltransferase*

The concentrations of Triton X-100 and digitonin giving the highest degree of activation of mouse-liver glucuronyltransferase are illustrated in Fig. 2. Bilirubin glucuronyltransferase exhibited the highest activity at 0.075% Triton X-100, while the activity towards the other substrates was highest at 0.050% Triton X-100. *o*-Aminophenol glucuronyltransferase was, however, not activated by Triton X-100, while 0.20% digitonin had a good effect.

*Activation of glucuronyltransferase by UDP-N-acetylglucosamine*

Enzyme maximally activated by preincubation could not be further activated by detergents, as also described earlier[5]. A similar reciprocal relationship exists between

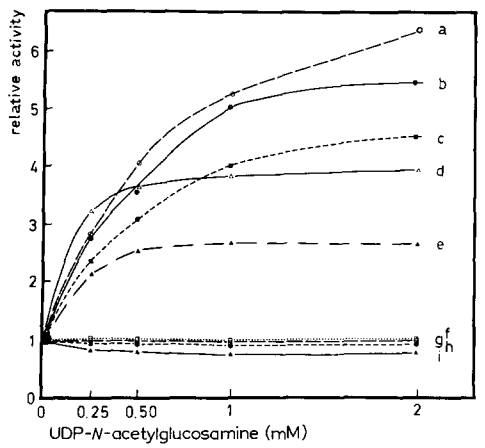


Fig. 3. Activation of glucuronyltransferase by UDP-*N*-acetylglucosamine. Mouse- and rat-liver suspensions (SI) were assayed for glucuronyltransferase activity with UDP-*N*-acetylglucosamine added to the incubation solutions in concentrations from 0 to 2.0 mM. The activities are given relative to the one where no UDP-*N*-acetylglucosamine was added. The Curves c and i represent rat-liver glucuronyltransferase activity assayed with *p*-nitrophenol without detergent (c) and with 0.05% Triton X-100 added (i). The other curves are from experiments with mouse-liver suspension. The Curves a, b, d, e and f represent enzyme activities when assayed without addition of detergent and with the following acceptor substrates: 4-methylumbelliferone (a), *p*-nitrophenol (b), phenolphthalein (d), *o*-aminophenol (e) and bilirubin (f). The enzyme activities for *p*-nitrophenol glucuronyltransferase with optimal concentration of Triton X-100 added to the incubate solutions are shown in Curve g, and Curve h represents the same enzyme activated by preincubation. Each curve represents one experiment.

UDP-*N*-acetylglucosamine activation and both detergent and spontaneous activation as shown in Fig. 3. UDP-*N*-acetylglucosamine added to the incubation solutions activated *p*-nitrophenol, *o*-aminophenol, 4-methylumbelliferone and phenolphthalein glucuronyltransferases in mouse and rat liver when fresh enzyme without detergent was assayed. When Triton X-100-activated or spontaneously activated enzyme were tested, however, no further activation was seen upon addition of UDP-*N*-acetylglucosamine. In the case of bilirubin glucuronyltransferase, activation by UDP-*N*-acetylglucosamine occurred neither in fresh enzyme preparations (rat and mouse) nor in detergent-activated preparations.

With 2 mM UDP-glucuronic acid in the incubation solutions, no activation was found by adding ATP[1] (0.25–4.0 mM) to native mouse liver when assayed for *p*-nitrophenol glucuronyltransferase, and no further activation was obtained by combining ATP and UDP-*N*-acetylglucosamine in contrast to earlier findings[1].

*The effect of diethylnitrosamine on rat-liver glucuronyltransferase*

The diethylnitrosamine activation of *o*-aminophenol glucuronyltransferase[8] was observed with both native enzyme and enzyme where detergents and UDP-*N*-acetylglucosamine were added as well (Table I). Enzyme with 0.05% Triton X-100 was activated 14.6 times by addition of diethylnitrosamine, resulting, however, in the same final activity as when digitonin and UDP-*N*-acetylglucosamine were combined with diethylnitrosamine. A slight activation of native 4-methylumbelliferone glucuronyltransferase was observed, whereas inhibition of the bilirubin enzyme occurred upon addition of diethylnitrosamine.

TABLE I

THE RELATION BETWEEN DIETHYLNITROSAMINE ACTIVATION OF GLUCURONYLTRANSFERASE AND ACTIVATION BY DETERGENTS AND UDP-*N*-ACETYLGLUCOSAMINE

Rat-liver suspension (SI) was assayed for glucuronyltransferase activity with bilirubin, *o*-aminophenol and 4-methylumbelliferone as substrates. Diethylnitrosamine, digitonin and UDP-*N*-acetylglucosamine were added to the incubation solutions in concentrations as indicated. The Triton X-100 concentrations used were 0.075% for bilirubin and 0.050% for *o*-aminophenol and 4-methylumbelliferone glucuronyltransferase. Other conditions were as described in MATERIALS AND METHODS. The values given are the mean of two separate experiments.

| *Conditions* | *Enzyme activity relative to that of native enzyme without additions* | | |
|---|---|---|---|
| | *Bilirubin* | *o-Aminophenol* | *4-Methylumbelliferone* |
| No additions | 1.00 | 1.00 | 1.00 |
| Triton X-100 in optimal concn. | 2.74 | 0.79 | 8.65 |
| 0.20% (w/v) digitonin | — | 2.68 | — |
| 2 mM UDP-*N*-acetylglucosamine | — | 2.49 | 4.10 |
| 15 mM diethylnitrosamine | 0.74 | 3.37 | 1.68 |
| Triton X-100 + 15 mM diethylnitrosamine | 2.10 | 11.53 | 8.60 |
| 0.20% digitonin + 15 mM diethylnitrosamine | — | 11.70 | — |
| 2 mM UDP-*N*-acetylglucosamine + 15 mM diethylnitrosamine | — | 11.65 | 3.15 |

*Kinetics of glucuronyltransferase*

With the exception of bilirubin glucuronyltransferase, typical hyperbolic UDP-glucuronic acid saturation curves were obtained when activated glucuronyltransferase was tested (Fig. 4). Bilirubin glucuronyltransferase activated by Triton X-100 exhibited, however, its highest activity at 2 mM UDP-glucuronic acid, showing slight inhibition at higher concentrations of UDP-glucuronic acid. The UDP-glucuronic acid saturation curves for nonactivated enzyme did not quite conform with the typical hyperbolic form and therefore did not give linear curves in Lineweaver–Burk plots. For this reason, the exact $K_m$ values for nonactivated enzyme cannot be determined. The apparent $K_m$ for native bilirubin and *o*-aminophenol glucuronyltransferases which exhibited $v_{max}$ at 0.5 mM of UDP-glucuronic acid was, however, lower than that for the activated enzymes. The curves for native 4-methylumbelliferone, *p*-nitrophenol, and phenolphthalein glucuronyltransferases did, however, show a slight steady rise from 1 to 8 mM UDP-glucuronic acid, so the actual $v_{max}$ cannot be predicted from Fig. 4.

The kinetics at varying acceptor substrate concentrations have only been investigated for *p*-nitrophenol glucuronyltransferase (Fig. 5). While the curve for fresh enzyme deviated from the linear course at higher concentrations of substrate, the curves for enzyme activated by Triton X-100, digitonin and UDP-*N*-acetylglucosamine all followed the straight line. The $K_m$ for Triton X-100-activated enzyme was 667 $\mu$M, while $K_m$ was 241 $\mu$M for enzyme activated by digitonin and UDP-*N*-acetylglucosamine. The last value was also found when prolonging the straight part of the curve for fresh enzyme.

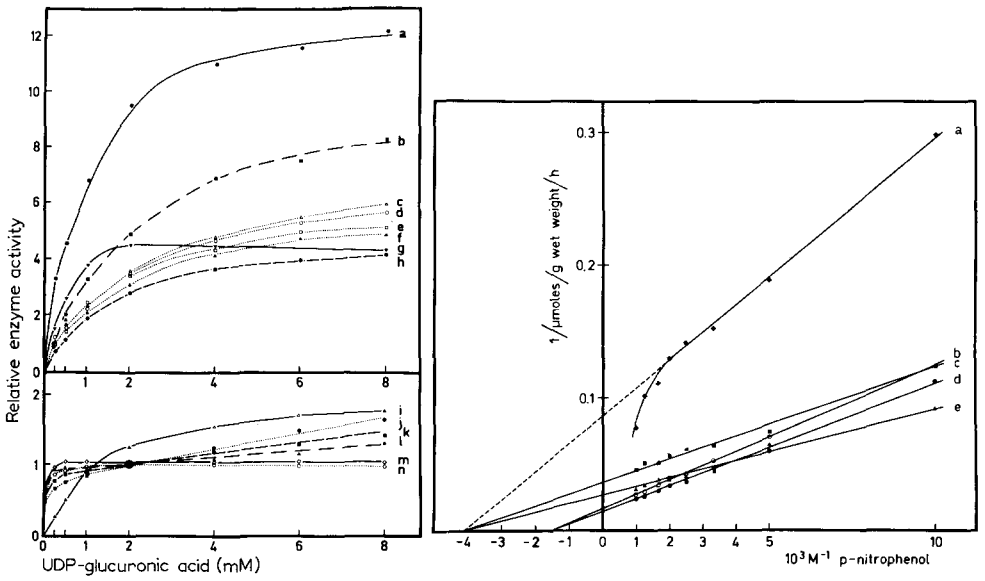


Fig. 4. UDP-glucuronic acid saturation curves for activated and nonactivated mouse-liver glucuronyltransferase. Mouse-liver glucuronyltransferase activity was measured with concentrations of UDP-glucuronic acid in the assay solutions varying from 0.25 to 8.0 mM. Blanks were enzyme suspensions incubated without addition of UDP-glucuronic acid. The Curves j, k, l, m and n represent fresh mouse-liver suspension (SI) assayed with 4-methylumbelliferone, phenolphthalein, *p*-nitrophenol, *o*-aminophenol and bilirubin, respectively. The other curves represent 4-methylumbelliferone with 0.05% Triton X-100 (a), phenolphthalein with 0.05% Triton X-100 (b), *p*-nitrophenol with enzyme activated by preincubation (c), *p*-nitrophenol with 0.05% Triton X-100 (d), *p*-nitrophenol with 0.10% digitonin (e), *p*-nitrophenol with 2 mM UDP-*N*-acetylglucosamine (f), bilirubin with 0.075% Triton X-100 (g) and *o*-aminophenol with 0.20% digitonin (i). The enzyme activities are given relative to the ones at 2 mM concentration of UDP-glucuronic acid with fresh enzyme without detergents or UDP-*N*-acetylglucosamine added. Each curve represents one experiment.

Fig. 5. The affinity between *p*-nitrophenol and glucuronyltransferase. Mouse-liver suspension (SI) was incubated with concentrations of *p*-nitrophenol varying from 0.1 to 1.0 mM. Other conditions were as described in MATERIALS AND METHODS except that the incubation time was 10 min. The curves represent fresh nonactivated enzyme (a), enzyme with 0.4% Triton X-100 (b), enzyme with 2 mM UDP-*N*-acetylglucosamine (c), enzyme with 0.05% Triton X-100 (d) and enzyme with 0.10% digitonin (e). Each curve represents the mean of two separate experiments.

*The pH optimum of glucuronyltransferase*

Fig. 6 shows that the pH optimum for detergent activated *p*-nitrophenol glucuronyltransferase was found at pH 6.2–6.6, while spontaneously activated enzyme exhibited the highest activity at pH 7.6–7.8. Native enzyme exhibited about the same activity in the range between pH 6.2 and 7.4. The pH-optimum curves for detergent-activated bilirubin, *o*-aminophenol, phenolphthalein and 4-methylumbelliferone glucuronyltransferases (Fig. 6) showed the same characteristics as those for non-activated enzyme (not shown in Fig. 6).

*The effect of varying relative amounts of protein and detergent*

As shown in Fig. 7, there was a linear relationship between enzyme activity and amount of enzyme protein up to a certain limit, although the detergent concentration

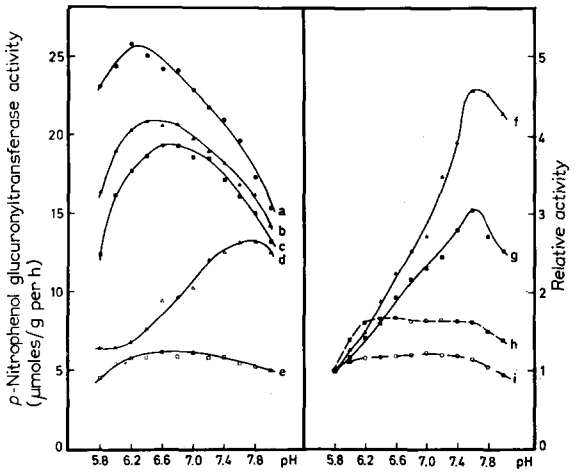


Fig. 6. The pH optimum of activated glucuronyltransferase. Mouse-liver suspension (SI) was assayed with 75 mM maleate buffer (pH 5.8–7.0) and with Tris–maleate buffer (pH 7.2–8.0). The Curves a, b, c and d show the activity of *p*-nitrophenol glucuronyltransferase when activated by 0.10% digitonin (a), 0.05% Triton X-100 (b), 0.025% deoxycholate (c) and preincubation at 0° for 5 days (d), while Curve e represents fresh nonactivated enzyme. The Curves f, g, h and i represent bilirubin with 0.075% Triton X-100 (f), *o*-aminophenol with 0.20% digitonin (g), phenolphthalein with 0.05% Triton X-100 (h), and 4-methylumbelliferone with 0.05% Triton X-100 (i). For the Curves f, g, h and i, activities are given relative to the ones at pH 5.8, while activities are given as μmoles per g wet wt. per h for *p*-nitrophenol glucuronyltransferase. The curves represent the mean of two experiments.

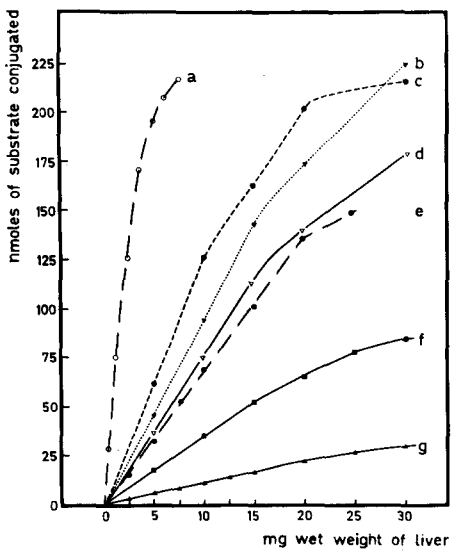


Fig. 7. The effect of the enzyme-protein concentration on the detergent activation. Variable amounts of mouse-liver suspension (SI) were incubated with constant concentration of detergent added to the assay solutions. The total amount of acceptor substrates present in the incubation solutions was always 250 nmoles. The curves represent 4-methylumbelliferone with 0.05% Triton X-100 (a), *p*-nitrophenol with 0.05% Triton X-100 (b), *p*-nitrophenol with 0.10% digitonin (c), *p*-nitrophenol with 0.025% deoxycholate (d), *p*-nitrophenol with enzyme activated by preincubation, without detergent present (e), *o*-aminophenol with 0.20% digitonin (f), bilirubin with 0.075% Triton X-100 (g). Most of the curves represent the mean of two experiments.

was kept constant. The curve representing *p*-nitrophenol glucuronyltransferase activated by preincubation, deviated from the linear course at the same level (about 50% of *p*-nitrophenol conjugated) as the curves for enzyme activated by detergents, indicating that shortage of substrate rather than of detergent may explain the deviation. These results are in some contrast to earlier observations[5], suggesting that activation depended on the relative amounts of detergent and microsomal phospholipids rather than simply on the concentration of detergent.

*Reversible inhibition of glucuronyltransferase by high concentrations of detergents*

Whereas mouse-liver *p*-nitrophenol glucuronyltransferase was strongly inhibited by 0.375% deoxycholate, this was completely reversed when after 30 min the enzyme detergent mixture was diluted giving a final deoxycholate concentration of 0.02% in the incubation solutions. The inhibition of mouse-liver 4-methylumbelliferone glucuronyltransferase by 3% Triton X-100 was likewise almost completely reversed upon dilution. Recovered activity in percent of the control was 92% after 1 h at 0° and 70% after 3 days at 0°. The control was identical to the test suspension except that detergent was not added until assay. These results are in contrast to those reported by LUEDERS AND KUFF[5], but in agreement with those reported by ERNSTER *et al.*[18] in the case of deoxycholate inhibition of NADH–cytochrome *c* reductase.

*Solubilization of glucuronyltransferase by Triton X-100*

In agreement with LUEDERS AND KUFF[5] no solubilization of glucuronyltransferase was obtained by preincubation. However, when mouse- and rat-liver suspensions were treated with Triton X-100, about 40% of the glucuronyltransferase activity towards all five acceptor substrates was recovered in the perfectly clear high-speed supernatant, (Table II). After storage of this fraction at 37° for 6 h, the remaining activity towards the different substrates in percent of the initial was about 5% for

TABLE II

SOLUBILIZATION OF MOUSE- AND RAT-LIVER GLUCURONYLTRANSFERASE BY TRITON X-100

Mouse- and rat-liver suspensions (SI) with different concentrations of Triton X-100 added was allowed to stand for 1 or 20 h at 0° before centrifugation at 40 000 × *g* for 120 min. The clear supernatant (SII) was carefully pipetted off leaving a pellet (PII) that was resuspended in a volume of 0.154 M KCl solution corresponding to the original volume of SI. The values given represents the mean of two parallels from one experiment.

| | *Percent of activity recovered in the soluble fractions, SII (SII + PII = 100%)* | | | | *Percent of activity in SI recovered in SII + PII* | |
|---|---|---|---|---|---|---|
| *Species* | *Mouse* | | | *Rat* | *Mouse* | *Rat* |
| *Triton X-100 concn.* | *0.125%* | *0.25%* | *0.50%* | *0.25%* | *0.25%* | *0.25%* |
| *Time before centrifugation* | *1 h* | *1 h* | *20 h* | *1 h* | *1 h* | *1 h* |
| Bilirubin glucuronyltransferase | 47 | 49 | 46 | 41 | 108 | 98 |
| 4-Methylumbelliferone glucuronyltransferase | 23 | 39 | 33 | 41 | 104 | 175 |
| *o*-Aminophenol glucuronyltransferase | 37 | 44 | 37 | 43 | 125 | 78 |
| *p*-Nitrophenol glucuronyltransferase | 43 | 43 | 38 | 49 | 94 | 114 |
| Phenolphthalein glucuronyltransferase | 32 | 44 | 44 | 48 | 83 | 108 |

bilirubin, 13% for *o*-aminophenol, 52% for *p*-nitrophenol, 74% for phenolphthalein and 80% for 4-methylumbelliferone. A similar stability pattern was obtained with the resuspended pellet, although the activity kept much better in this fraction.

*The degree of activation of glucuronyltransferase in different tissues*

Similar activation factors for glucuronyltransferase were found in liver compared with kidney and in newborn (less than 24 h old) compared with adult rat tissues. Earlier conclusions on the relative glucuronyltransferase activities in these tissues are then still valid.

*Hydrolysis of the glucuronyl conjugates*

The methods based on reduction in absorbance and fluorescence were checked by incubation of the enzymic reaction products with β-glucuronidase (see MATERIALS AND METHODS). 90–100% hydrolysis was obtained, and this was totally inhibited by saccharo-1,4-lactone (specific inhibitor of β-glucuronidase[19]).

## DISCUSSION

Since the activation of glucuronyltransferase by preincubation, detergents and UDP-*N*-acetylglucosamine is of a similar magnitude, is nonadditive and has the same effect on the UDP-glucuronic acid saturation curves, it is considered reasonable to seek a common principle underlying this activation.

No evidence has been found for the existence of an inhibitor of glucuronyltransferase in fresh liver suspensions by experiments where nonactivated and spontaneously activated preparations were combined, in agreement with LUEDERS AND KUFF[5]. Decreased β-glucuronidase activity, thought to play some role in the activation observed by ISSELBACHER *et al.*[2], was not observed in the activated preparations (Fig. 1).

The somewhat atypical curves for nonactivated enzyme in Figs. 4 and 5 may not reflect only the affinity between the enzyme and its substrates but perhaps other as yet unknown phenomenons in addition. In spite of this reservation, it seems reasonable to assume that the activated enzyme does not exhibit better kinetic properties than nonactivated enzyme. That the curves were made typical upon activation may indicate that the postulated interfering phenomenon is just what has to be "removed" during the process of activation.

The activation of glucuronyltransferase might be explained if one form of the enzyme were nonfunctioning in native preparations because its active site was inaccessible for the substrates. This masking of the active site might, for instance, be brought about by a special steric configuration of the enzyme or by attachment of a masking substance. Both detergents and preincubation are known to cause dispersion of membraneous material[18,20] and therefore might be thought to unmask the active site(s).

POGELL AND LELOIR[1] described the activating effect of UDP-*N*-acetylglucosamine and ATP on *p*-nitrophenol glucuronyltransferase. Mostly this effect was thought to be due to protection of UDP-glucuronic acid against breakdown by pyrophosphatase, although some as yet unexplained effect remained even at saturating concentrations of UDP-glucuronic acid (8.7 mM). The evidence against inhibition of UDP-glucuronic

acid breakdown as the mechanism of activation by detergents and preincubation is discussed by LUEDERS AND KUFF[5]. The activation of *p*-nitrophenol glucuronyltransferase by UDP-*N*-acetylglucosamine at varying UDP-glucuronic acid concentrations (0.25–8.0 mM), was quite similar to that by detergents and preincubation (Fig. 4).

UDP-*N*-acetylglucosamine being a substrate analogue might be an allosteric activator of glucuronyltransferase, resulting in the same unmasking of the active site as that obtained with detergents and preincubation. The effect of UDP-*N*-acetylglucosamine most probably is quite specific compared with that of detergents and preincubation. This may be the reason why bilirubin glucuronyltransferase was not activated by UDP-*N*-acetylglucosamine, since evidence indicates that bilirubin is conjugated by a separate enzyme (stability experiments and ref. 2).

The activation of glucuronyltransferase by snake venom[2,3] is probably analogous to the detergent activation since solubilization takes place in both cases[2,7]. The activation of glucuronyltransferase by dialysis overnight[4] may be related to spontaneous activation.

Since diethylnitrosamine activated *o*-aminophenol glucuronyltransferase where detergents and UDP-*N*-acetylglucosamine were added as well, the effect of diethylnitrosamine must be unrelated to that of the other activating principles discussed. That the same final activity was obtained whether Triton X-100, digitonin, or UDP-*N*-acetylglucosamine were added together with diethylnitrosamine, is another indication that detergents and UDP-*N*-acetylglucosamine act in the same way. It is unknown why Triton X-100 alone did not activate *o*-aminophenol glucuronyltransferase although good effect was seen when combined with diethylnitrosamine (Table I). Triton X-100 also made the UDP-glucuronic acid saturation curve for *o*-aminophenol glucuronyltransferase hyperbolic (Fig. 4i), and solubilized 40% of the *o*-aminophenol enzyme (Table II).

The differences in the time-course and variability in degree of activation seen in Fig. 1 is most easily explained by there being a multiplicity of glucuronyltransferases.

The question of the true glucuronyltransferase activity *in vivo* is a difficult one. Besides the amount of enzyme and UDP-glucuronic acid, the accessibility of substrates for the active site may be important in regulating the function of the glucuronyl-conjugating system. Probably, however, a good answer about the amount of enzyme is obtained by assaying the enzyme activity with optimal concentration of detergent in the incubation solutions. Only slight modifications of the assay methods were necessary when estimating detergent-activated glucuronyltransferase, as have been described in this paper.

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