Possible Multiple Binding Sites for *o*-Aminophenol on Uridine Diphosphate Glucuronyltransferase

By RICHARD D. HOWLAND and LINDA D. BOHM

*Department of Pharmacology, New Jersey Medical School, College of Medicine and
Dentistry of New Jersey, Newark, NJ 07103, U.S.A.*

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1. Hepatic microsomal UDP-glucuronyltransferase (EC 2.4.1.17) derived from either weanling or adult rats exhibits three pH optima, at pH 5.4, 7.2 and 9.2, when *o*-aminophenol is the acceptor substrate, whereas when *p*-nitrophenol is the acceptor substrate only one pH optimum is observed, at pH 5.4. 2. Prior treatment of rats of either age with 3-methylcholanthrene results in a 2–3-fold increase in *o*-aminophenol conjugation at pH 5.4 and a 6–9-fold increase at pH 9.2. At pH 7.2, the induced enzyme is 2 to 3 times more active towards *o*-aminophenol than the control enzyme, but no pH optimum is demonstrable. 3. *o*-Aminophenol conjugation at pH 5.4 and 9.2 is inhibited competitively by both *p*-nitrophenol and *p*-nitrophenyl glucuronide, suggesting that the two phenolic aglycones share the same binding site. At pH 7.2, however, *p*-nitrophenyl glucuronide does not inhibit *o*-aminophenol conjugation, suggesting that the binding site at this pH is not shared by the two phenols. These data are consistent with the existence of more than one binding site for *o*-aminophenol on UDP-glucuronyltransferase.

The pH-optimum curve for hepatic glucuronyltransferase (UDP-glucuronosyltransferase, EC 2.4.1.17) has been reported as having a single peak ranging between pH 6.6 and 8.2, depending on the substrate used for assay (Dutton, 1966). Howland & Burkhalter (1971) have shown that glucuronyltransferase activity of rat hepatic microsomal fraction exhibits an optimum at pH 9.2 when assayed with *o*-aminophenol in both 3-methylcholanthrene-induced and control animals. Since reported values for the pH optimum with this substrate have ranged between pH 7.3 and 7.9 (Dutton, 1966), we decided to reinvestigate the pH-dependent behaviour of glucuronyltransferase towards *o*-aminophenol and *p*-nitrophenol in both control and 3-methylcholanthrene-treated rats. The latter substrate was included since it has been shown that it does not share a common binding site with *o*-aminophenol (Zakim *et al.*, 1973).

The evidence in the present work indicates that rat glucuronyltransferase exhibits three pH optima towards *o*-aminophenol and that these optima represent different binding sites for *o*-aminophenol, as indicated by kinetic behaviour, mixed-substrate interaction and effect of 3-methylcholanthrene.

A preliminary report of part of this work has been published (Howland, 1975).

Materials and Methods

Chemicals

o-Aminophenol was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. *p*-Nitrophenol and

3-methylcholanthrene were obtained from Calbiochem, Los Angeles, CA, U.S.A. D-Glucaric acid 1,4-lactone (glucarolactone), *p*-nitrophenyl glucuronide and UDP-glucuronic acid were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Authentic *o*-aminophenyl glucuronide was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. All other chemicals were reagent grade and obtained from local suppliers.

Animals and tissue preparation

Male Wistar rats, either weanling (40–50 g) or adult (200–300 g), were purchased from Marland Breeding Farms, Hewitt, NJ, U.S.A. Animals were allowed free access to food and water during the experimental period. Rats were divided into two groups: the treated group was injected with 3-methylcholanthrene dissolved in corn oil (40 mg/kg; intraperitoneally) once a day for 3 days, and the control group received an equivalent volume of corn oil once a day for 3 days.

At 24 h after the last injection, the animals were killed by decapitation and their livers rapidly removed, blotted and weighed. All subsequent steps were performed at 2–5°C. Livers were minced and homogenized in 3 vol. (w/v) of cold 1.15% KCl in a Potter-Elvehjem homogenizer (Teflon pestle). Homogenates were centrifuged at 9000 *g*_{av} for 20 min in a Lourdes model A refrigerated centrifuge. The resulting supernatant was further centrifuged at 100000 *g*_{av} for 60 min in a Beckman L2 ultracentrifuge. After this high-speed centrifugation, the supernatant was carefully decanted and the

microsomal pellet was resuspended in 1.15% (w/v) KCl by using a Dounce homogenizer, such that 1 ml of the final suspension contained the microsomal fraction derived for either 0.5 or 1.0 g of liver.

Enzyme assays

Typical incubation mixtures contained the following final concentrations of reactants: 1–3 mg of protein; 5.0 mM-MgCl₂; 5.0 mM-glucuronalactone to inhibit β -glucuronidase; 2.0 mM-UDP-glucuronic acid; 1.0 mM-*o*-aminophenol or 0.4 mM-*p*-nitrophenol;

40 mM of the appropriate buffer in a final volume of 1.0 ml. Buffers were sodium acetate/acetic acid, pH 3.8–5.6; Tris/maleate, pH 5.2–8.6; glycine/NaOH, pH 8.6–12.8. All reactants except UDP-glucuronic acid were preincubated together for 3 min, the reaction was started by addition of UDP-glucuronate and the incubation allowed to proceed at 37°C for 15 min in a Dubnoff metabolic shaking incubator. The actual pH was determined at 37°C in flasks containing all reactants.

In the kinetic studies, the final volume was

Fig. 1. Effect of pH on *o*-aminophenol conjugation

Microsomal fractions derived from the livers of two or three rats were assayed for glucuronyltransferase activity towards *o*-aminophenol at various H⁺ concentrations. Buffers were sodium acetate/acetic acid (●), Tris/maleate (○) and glycine/NaOH (△). (a) Weanling control; (b) weanling 3-methylcholanthrene-treated; (c) adult control; (d) adult 3-methylcholanthrene-treated animals.

decreased to 0.5ml and all reactants were kept at the same concentration except as noted. Experimental conditions were such that rates of conjugation were linear with time and protein concentration.

When *o*-aminophenol was the substrate, the reaction was terminated by the addition of an equal volume of 1.0M-trichloroacetic acid/1.0M-KH₂PO₄ mixture, pH2.25. The vessels were placed in the cold for 30min, and then centrifuged at 2000rev./min for 10min in an International centrifuge (model K) to precipitate protein. The formation of *o*-aminophenyl glucuronide was quantified by the method of Dutton & Storey (1962).

When *p*-nitrophenol was the substrate, the reaction was stopped by the addition of 1 vol. of 1.0M-trichloroacetic acid. The samples were centrifuged as above and the disappearance of *p*-nitrophenol was determined by adding 0.8ml of water

and 0.1ml of 10M-KOH to a 0.2ml sample and measuring the concentration of *p*-nitrophenol remaining (Isselbacher, 1956).

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

Statistical significance between means was tested by Student's *t* test.

Results

Effect of H⁺ concentration on o-aminophenol conjugation

The activity of UDP-glucuronyltransferase towards *o*-aminophenol, when determined over the pH range 4–10, exhibited not one but three pH optima in both weanling and adult rats (Figs. 1a and 1c). The optima were relatively sharp and occurred at

Fig. 2. *Effect of pH on p-nitrophenol conjugation*

Microsomal fractions derived from the livers of two or three rats were assayed for glucuronyltransferase activity towards *p*-nitrophenol at various H⁺ concentrations. Buffers were sodium acetate/acetic acid (●), Tris/maleate (○) and glycine/NaOH (Δ). (a) Weanling control; (b) weanling 3-methylcholanthrene-treated; (c) adult control; (d) adult 3-methylcholanthrene-treated animals.

pH 5.4, 7.2 and 9.2. In weanlings the activities at pH 5.4 and 9.2 were about twice that at pH 7.2. On the other hand, the optima in the adult were all approximately the same, ranging between 3 and 4 nmol/15 min per mg of protein.

When the activity of the enzyme from 3-methylcholanthrene-treated rats was determined over the same pH range, both quantitative and qualitative differences were observed (Figs. 1*b* and 1*d*). The optima at pH 5.4 and 9.2 were present, but the optimum at pH 7.2 was no longer observed. Instead, activity was constant over the pH range 6.0–8.0, although it was much greater than that of control animals, nearly 2-fold in weanlings and 3-fold in adults. It is noteworthy that, on comparison of the effect of 3-methylcholanthrene treatment on the glucuronyltransferase activity at pH 9.2 and 5.4, there was a relatively greater increase in activity at the lower H^+ concentration. In weanling rats the respective increases in activity at each optimum were 6- and 2-fold, and in adults they were 9- and 4-fold.

*Effect of H^+ concentration on *p*-nitrophenol conjugation*

In contrast with the conjugation of *o*-aminophenol, UDP-glucuronyltransferase activity towards *p*-nitrophenol exhibited only one pH optimum, at pH 5.1 in weanlings (Fig. 2*a*) and at pH 5.4 in adults (Fig. 2*c*). The specific activity was much higher than that for *o*-aminophenol, being 76 and 96 nmol/15 min per mg of protein in weanling and adult rats respectively. In both age groups, however, significant but constant activity was observed between pH 6.0 and 9.5, of approximately one-third of the optimal value.

Treatment of weanling rats with 3-methylcholanthrene resulted in an increase in enzyme activity at the pH optimum, from 76 to 184 nmol/15 min per mg of protein (Fig. 2*b*). In addition, activity at all other pH values was increased 2.5-fold. However, in adult rats treated with 3-methylcholanthrene, no increase in activity at the pH optimum was observed (Fig. 2*d*). Yet at the pH usually used for assay (pH 7.4), the enzyme activity was 50% greater than the control activity (46 compared with 31 nmol/15 min per mg of protein). As the H^+ concentration decreased, enzyme activity continued to increase, so that at pH 9.2 62 nmol/15 min per mg of protein was conjugated by liver from induced animals compared with 36 nmol/15 min per mg in control livers.

Effect of acid and alkaline pH pretreatment on UDP-glucuronyltransferase activity

The multiple pH optima exhibited towards *o*-aminophenol may have been due to an irreversible activation of the enzyme, as has been reported (Vessey & Zakim, 1971; Marniemi & Vainio, 1976). To test this, we preincubated microsomal fractions at

acid and alkaline pH, in both the presence and the absence of *o*-aminophenol, and then determined the effect on enzyme activity assayed at pH 7.4 (Table 1). No activation of *o*-aminophenol conjugation was observed after preincubation at either pH 5.4 and 9.2, whether or not the aglycone was present. In addition, preincubation of microsomal fractions with 1.1 mM-ascorbic acid (added to lessen oxidation of *o*-aminophenol) did not affect *o*-aminophenol conjugation.

In a similar study with *p*-nitrophenol (Table 1), no activation was observed, although preincubation with the aglycone resulted in approx. 50% decrease in the observed activity. Again, the effects were independent of the pH at which the microsomal fractions were incubated.

*Kinetic behaviour of *o*-aminophenyl UDP-glucuronyltransferase*

Another explanation of the multiple pH optima exhibited by glucuronyltransferase towards *o*-aminophenol was that more than one enzyme was responsible for the conjugation of this substrate. In an effort to answer this question, the active site of

Table 1. *Effect of acid or alkaline pH pretreatment on UDP-glucuronyltransferase activity at pH 7.4*

Microsomal fractions were adjusted to the indicated pH and preincubated for 30 min at 0°C before returning them to pH 7.4 for assay. In addition to substrate and enzyme, all assay media contained 2.0 mM-UDP-glucuronic acid, 50 mM-Tris/HCl buffer, pH 7.4, and 5 mM-MgCl₂. Activity in fractions preincubated at pH 7.0, with no additions, was taken as 100% and equalled 4.92 nmol of *o*-aminophenol glucuronide formed/15 min per mg of protein and 12.5 nmol of *p*-nitrophenol glucuronide formed/15 min per mg of protein.

Preincubation conditions	Activity at pH 7.4 (%)
Expt. 1 (1.0 mM- <i>o</i> -aminophenol)	
pH 7.0, no additions	100
pH 7.0+1.1 mM-ascorbate	91
pH 7.0+1.0 mM- <i>o</i> -aminophenol	112
pH 5.4, no additions	105
pH 5.4+1.1 mM-ascorbate	104
pH 5.4+1.0 mM- <i>o</i> -aminophenol	103
pH 9.2, no additions	113
pH 9.2+1.1 mM-ascorbate	106
pH 9.2+1.0 mM- <i>o</i> -aminophenol	97
Expt. 2 (0.4 mM- <i>p</i> -nitrophenol)	
pH 7.0, no additions	100
pH 7.4+0.4 mM- <i>p</i> -nitrophenol	56
pH 5.4, no additions	113
pH 5.4+0.4 mM- <i>p</i> -nitrophenol	63
pH 9.2+no additions	109
pH 9.2+0.4 mM- <i>p</i> -nitrophenol	50

o-aminophenol glucuronyltransferase was characterized in terms of its interaction with *o*-aminophenol and *p*-nitrophenol, the latter compound being chosen since it was conjugated over the entire pH range. In addition, these studies were performed on microsomal fractions from adult rats. When various concentrations of *o*-aminophenol were incubated at pH 9.2 with 8 mM-UDP-glucuronate (nearly saturating) and either 0.05 or 0.2 mM-*p*-nitrophenol and a double-reciprocal plot was made of the data, the results indicated that *p*-nitrophenol competitively inhibited *o*-aminophenol conjugation, with an apparent K_i of 0.10 mM (Fig. 3a). To eliminate effects of competition for UDP-glucuronate, a similar experiment was performed by substituting 10 mM-*p*-nitrophenyl glucuronide for the aglycone (Fig. 3b). This glucuronide was also a competitive inhibitor, with an apparent K_i of 23.5 mM. As suggested by Zakim *et al.* (1973), if two substrates bind to the same

active site of glucuronyltransferase then it should be possible to demonstrate product inhibition, by the glucuronide of one of the substrates, of the conjugation of the alternative substrate. Thus the results suggest that at pH 9.2 *o*-aminophenol and *p*-nitrophenol share a common binding site.

When the interaction of *o*-aminophenol and *p*-nitrophenyl glucuronide was studied at pH 5.4, qualitatively similar results were obtained. As summarized in Table 2, both *p*-nitrophenyl glucuronide and its aglycone competitively inhibited *o*-aminophenol conjugation, again suggesting a common binding site for the two phenols.

It should be pointed out, however, that differences in the kinetic behaviour of *o*-aminophenol glucuronyltransferase at pH 5.4 and 9.2 were evident after 3-methylcholanthrene administration (Table 2). When assayed at pH 9.2, *o*-aminophenol glucuronyltransferase from induced rats exhibited a significantly

Fig. 3. Kinetic behaviour of *o*-aminophenol glucuronyltransferase at pH 9.2

The double-reciprocal plots shown were obtained under conditions described in the text. (a) Glucuronyltransferase activity towards *o*-aminophenol, alone (●), in the presence of 0.05 mM-*p*-nitrophenol (○) and in the presence of 0.02 mM-*p*-nitrophenol (■). (b) Glucuronyltransferase activity towards *o*-aminophenol, alone (●), and in the presence of 10 mM-*p*-nitrophenyl glucuronide (○). For all curves v is expressed as nmol of glucuronide formed/min per mg of microsomal protein.

Table 2. *Michaelis constants of o-aminophenol glucuronyltransferase determined at various H⁺ concentrations*

Microsomal fractions from either control or 3-methylcholanthrene-treated rats were incubated at the indicated pH along with various concentrations of *o*-aminophenol: from 0.25 to 1.0 mM at pH 9.2 and from 0.25 to 1.5 mM at pH 5.4. The K_i values were obtained by using the following concentrations of inhibitors: 0.05 mM-*p*-nitrophenol or 10 mM-*p*-nitrophenyl glucuronide. Constants are means $\pm$ S.E.M. of determinations from three animals where indicated. All other values are single estimates from either one or two animals.

Treatment	pH of assay	V_{max} (nmol/min per mg of protein)	K_m (mM)	K_i (mM)	
				<i>p</i> -Nitrophenol	<i>p</i> -Nitrophenyl glucuronide
Control	9.2	3.72 ± 0.27	0.84 ± 0.08	0.09, 0.12	18.3, 23.5
3-Methylcholanthrene	9.2	$8.62 \pm 0.46^*$	$0.46 \pm 0.04^*$	0.044, 0.049	Not determined
Control	5.4	3.39 ± 0.41	5.08 ± 1.00	0.058	3.4
3-Methylcholanthrene	5.4	$6.94 \pm 0.43^*$	5.63 ± 1.03	0.067	Not determined

* $P < 0.05$ compared with corresponding control.

Fig. 4. Kinetic behaviour of *o*-aminophenol glucuronyltransferase at pH 7.2

The double-reciprocal plot shown was obtained under conditions described in the text. The points represent either *o*-aminophenol alone ($\circ$) or in the presence of 10 mM-*p*-nitrophenyl glucuronide ($\bullet$). On the ordinate, v is expressed as nmol of glucuronide formed/min per mg of microsomal protein.

lower apparent K_m for *o*-aminophenol as compared with control rats ($P < 0.05$), and the K_i for *p*-nitrophenol was also decreased. In contrast, at pH 5.4 the K_m for *o*-aminophenol and the K_i for *p*-nitrophenol were unchanged from control values.

As expected, the V_{max} of the induced enzyme was significantly greater at both pH optima.

The active site of *o*-aminophenol glucuronyltransferase assayed at pH 7.4 differed significantly from those at pH 5.4 and 9.2. When the effect of 10 mM-*p*-nitrophenyl glucuronide on the conjugation of *o*-aminophenol was studied, it was found that this glucuronide did not have any effect on the rate of *o*-aminophenyl glucuronide formation (Fig. 4). The results suggest that *o*-aminophenol and *p*-nitrophenol do not share common binding sites on glucuronyltransferase when assayed at pH 7.2. Similar kinetic studies by Sanchez & Tephly (1974) and Zakim *et al.* (1973) have distinguished between synthesis of *p*-nitrophenyl glucuronide and morphine glucuronide, and synthesis of *p*-nitrophenyl glucuronide and *o*-aminophenyl glucuronide respectively.

Discussion

To our knowledge, multiple pH optima for UDP-glucuronyltransferase have not been previously reported. For *o*-aminophenol in particular, a single pH optimum ranging between pH 7.3 and 7.9 has been found in different species and tissues (Dutton, 1966). The optimum at pH 7.2 for *o*-aminophenol reported here corresponds to the published values. In addition, the optimum at pH 9.2 confirms that found by Howland & Burkhalter (1971). However, the optimum at pH 5.4 exhibited by glucuronyltransferase towards both *o*-aminophenol and *p*-nitrophenol is well below that of any known value (Dutton, 1966). The inclusion of glucarolactone in the incubation mixture eliminates any possible participation of β -glucuronidase in the formation of these glucuronides. It is also unlikely that products other than *o*-aminophenyl glucuronide were measured, since (a) omission of UDP-glucuronate from the reaction mixture resulted in no characteristic colour formation and (b) the

conditions of the colour formation are such that *o*-aminophenyl sulphate and the free phenol do not react (Dutton & Storey, 1962).

Since *o*-aminophenol is a zwitterion, having two ionizable groups, namely a phenolic hydroxyl, pK_a9.7, and a primary amine, pK_a4.7 (von Eggers *et al.*, 1954), it is possible that changes in the ionization of these groups was a factor in determining the pH optima. The theoretical proportion of uncharged and charged species was calculated at each of the pH optima, i.e. pH5.4, 7.2 and 9.2. For the phenolic hydroxyl group, the percentage of the molecules un-ionized is 99.95, 99.7 and 76.0 at each respective optimum, and for the primary amine, the respective percentage un-ionized is 88.3, 99.7 and 99.997. These data indicate that the pH optima for *o*-aminophenol do not reflect large changes in the degree of ionization of this substrate, and the observed optima may be due to changes in the ionization of the amino acids in the active site of the enzyme protein. The data also suggest that it is the un-ionized form of the substrate that is bound to the active site. It is noteworthy that at the observed pH optimum of 5.4 for *p*-nitrophenol (pK_a7.15) 98.2% of the molecules are undissociated, also suggesting that it is the un-ionized species that is bound by the enzyme.

It is well known that the phospholipid environment can markedly influence the apparent activity of UDP-glucuronyltransferase, as shown by the reports on the effect of phospholipase A (Vessey & Zakim, 1971) and detergents (Winsnes, 1969, 1971; Howland *et al.*, 1971). In the present report, treatment of microsomal fractions at alkaline or acid pH reversibly activated UDP-glucuronyltransferase, which is in contrast with the reports of irreversible activation of the enzyme activity occurring at about pH10 (Vessey & Zakim, 1971; Marniemi & Vainio, 1976). The activation we observed may have been brought about by reversible changes in the phospholipid environment, resulting in increased substrate availability or decreased enzyme constraint. Significantly, UDP-glucuronyltransferase from microsomal fractions treated with Lubrol exhibits only a single pH optimum towards *o*-aminophenol and *p*-nitrophenol (Howland *et al.*, 1971), suggesting that the micro-environment is important in determining the pH optima. Alterations in the membrane components may also account for the disappearance of the pH optimum at pH7.2 after 3-methylcholanthrene treatment.

Although some interaction between H⁺ concentration and non-enzymic components of the microsomal membrane cannot be ruled out as a factor affecting UDP-glucuronyltransferase activity, the results of the kinetic experiments on *o*-aminophenol-binding sites strongly suggest that they are not identical. The data are consistent with the hypothesis that

p-nitrophenol binds to the same site as *o*-aminophenol at pH5.4 and 9.2, whereas at pH7.2 the binding sites for the two substrates appear to be mutually exclusive. The absence of inhibition observed at pH7.2 with *p*-nitrophenyl glucuronide is not related to the reported stimulatory effect of glucuronic acid derivatives on UDP-glucuronyltransferase (Vessey & Zakim, 1974), since this phenomenon occurs at UDP-glucuronic acid concentrations less than 2–4 mM, whereas the concentration used in the present study was 8 mM. It is noteworthy that the other mutually exclusive binding sites reported for this enzyme also occur in the same pH range (Sanchez & Tephly, 1974; Zakim *et al.*, 1973). We do not know whether or not the binding sites represent different forms of the enzyme, although Del Villar *et al.* (1975) have physically separated morphine glucuronyltransferase and *p*-nitrophenol glucuronyltransferase by means of DEAE-cellulose chromatography.

Since the activity of UDP-glucuronyltransferase is greatly influenced by membrane components in 'native' microsomal fractions, the effects of pH and substrate interactions in detergent-treated microsomal fractions need to be studied in more detail.

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