Defective function of a microsomal UDP-glucuronyltransferase in Gunn rats

(p-nitrophenol/kinetic parameters/glucuronidation/reverse reaction/multiplicity of glucuronyltransferase)

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ABSTRACT   The kinetic parameters of the p-nitrophenol-metabolizing form of UDP-glucuronyltransferase [=UDPglucuronosyltransferase; UDPglucuronate β-glucuronosyltransferase (acceptor-unspecific), EC 2.4.1.17] have been compared in liver microsomes from the Gunn strain of rat and from normal, Wistar rats. The abnormally low rate of glucuronidation of p-nitrophenol in the Gunn rats, as compared with Wistar rats, is due to decreased affinity of UDP-glucuronyltransferase for UDP-glucuronic acid. Activities at V_{max} and the Michaelis constant for p-nitrophenol, K_{PNP}, of UDP-glucuronyltransferase are the same for enzyme from either strain of rat. Studies of the kinetic parameters of the reverse reaction catalyzed by UDP-glucuronyltransferase indicate that the enzyme from Gunn rats also has decreased affinity for UDP. Calculated values of $\Delta G°$ for the binding of the UDP portion of UDP-glucuronic acid suggest that the defect of UDP-glucuronyltransferase of Gunn rats appears limited to abnormal interactions between the enzyme and the UDP portion of UDP-glucuronic acid. Studies of the extent of UDP-induced inhibition of the forward reaction support this idea. Diethylnitrosamine, added to microsomes in vitro, enhances the affinity of UDP-glucuronyltransferase for the UDP portion of UDP-glucuronic acid. Despite the defective conformation of the UDP-glucuronic acid binding site of UDP-glucuronyltransferase from Gunn rats this enzyme is activated in the normal way by UDP-N-acetylglucosamine, which is a K-type effector with regard to UDP-glucuronic acid.

Homozygous rats of the Gunn strain have a genetically determined deficiency in their capacity to synthesize glucuronides (1–3). The extent of the abnormality varies with different aglycones. Thus, there is no detectable activity of UDP-glucuronyltransferase [=UDPglucuronosyltransferase; UDPglucuronate β-glucuronosyltransferase (acceptor-unspecific) EC 2.4.1.17] assayed with bilirubin as aglycone in liver microsomes from Gunn rats, but rates of glucuronidation of o-aminophenol and o-aminobenzoic acid are about 20% of normal (2–4). The rate of glucuronidation of p-nitrophenol in liver microsomes from Gunn rats is decreased only slightly as compared with normal rats (4, 5). Since there is kinetic evidence that bilirubin, p-nitrophenol, o-aminophenol, and o-aminobenzoic acid are glucuronidated at separate active sites (6–8), it is reasonable to conclude that there are several defective forms of UDP-glucuronyltransferase in Gunn rats. The exact nature of the abnormality in the function of UDP-glucuronyltransferases in Gunn rats is thus an interesting and provocative problem. This question also has practical importance in view of the occurrence in man of a biochemi-

cal equivalent of the disease of Gunn rats (2). We have been interested, therefore, in the type of kinetic abnormality in the function of UDP-glucuronyltransferase in liver microsomes from Gunn rats. Data presented in this paper lead us to conclude that the p-nitrophenol-metabolizing form of UDP-glucuronyltransferase from Gunn rats, as compared with normal rats, has decreased affinity for the UDP portion of UDP-glucuronic acid. Affinity of the abnormal enzyme for the aglycone is unaltered, as are activities at V_{max} of the forward and reverse reactions.

MATERIALS AND METHODS

Homozygous male Gunn rats were from a colony maintained by Dr. Michael Thaler at the University of California, San Francisco. Male Wistar rats were purchased from Simonsen Laboratories. Liver microsomes were prepared as described previously (9), and stored at −20° for up to 3 weeks.

Liver microsomes from rats, and several other species, contain a nucleotide pyrophosphatase which catalyzes the hydrolysis of UDP-glucuronic acid (10). The activity of the microsomal nucleotide pyrophosphatase was measured in preliminary studies and found to be extremely low in the microsomal preparations used in the present experiments. Less than 1% of a 1 mM solution of UDP-glucuronic acid was hydrolyzed by this enzyme under conditions used for the assay of UDP-glucuronyltransferase. The pyrophosphatase hence did not interfere with measurements of initial rates of the activity of UDP-glucuronyltransferase.

The activity of the forward reaction catalyzed by UDP-glucuronyltransferase was assayed at 37° with p-nitrophenol as aglycone in 50 mM Tris·HCl, pH 7.4. Initial rates of activity were determined as in ref. 8. Assays were carried out under conditions for which the kinetics are Michaelis–Menten in form (7, 8). The concentrations of substrates are given in the legends to the figures and tables. All assays contained 5 mM EDTA in order to remove trace amounts of divalent metal ions which are activators of UDP-glucuronyltransferase when the forward reaction is assayed (8), but which inhibit the rate of the reverse reaction (11). The effects of metal ions were the same when measured with microsomes from Gunn or Wistar rats.

The conditions for measuring initial rates of the reverse reaction were identical to those used for the forward reaction except that saccharic acid-1,4-lactone was added to inhibit β-glucuronidase (12). The concentrations of UDP and p-nitrophenylglucuronic acid, substrates of the reverse reaction, are indicated in the legends to figures and tables. Protein concentration was determined by the biuret method (13).

Abbreviations: UDPGA, UDP-glucuronic acid; PNP, p-nitrophenol; PNPGA, p-nitrophenylglucuronic acid.
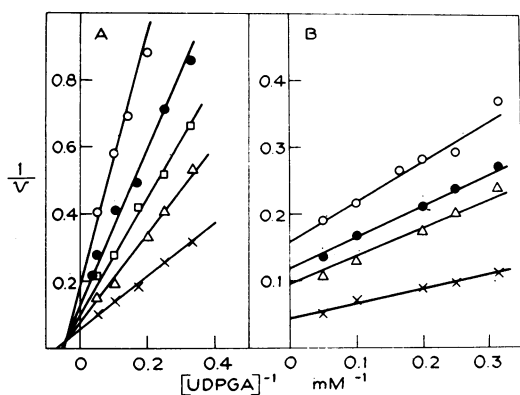* To whom reprint requests should be addressed at the Molecular Biology Division, Veterans Administration Hospital.

FIG. 1. Double reciprocal plots of the rate of glucuronidation of p-nitrophenol as a function of the concentration of UDP-glucuronic acid (UDPGA) in microsomes from rats of the Gunn strain (A) and normal, Wistar rats (B). Initial rates of activity of UDP-glucuronyltransferase were measured, as in *Materials and Methods*, with the indicated concentrations of UDP-glucuronic acid at different fixed concentrations of p-nitrophenol: 0.06 mM (O); 0.1 mM (●); 0.14 mM (□); 0.2 mM (△); and 0.4 mM (X). Units of activity are nmol of p-nitrophenol glucuronidated per min/mg of protein.

RESULTS

Kinetic parameters of UDP-glucuronyltransferase in liver microsomes from Gunn rats and Wistar rats

Double reciprocal plots of the rate of glucuronidation of p-nitrophenol as a function of the concentration of UDP-glucuronic acid, at several fixed concentrations of p-nitrophenol, are shown for liver microsomes from Gunn and Wistar rats in Fig. 1. Secondary plots used for graphical determination of activity at V_{max} are in Fig. 2. Since UDP-glucuronyltransferase has a random order, rapid equilibrium kinetic mechanism (14), the K terms in Table 1 are the dissociation constants for enzyme–UDP-glucuronic acid and enzyme–p-nitrophenol complexes (15, 16). The defect in the p-nitrophenol-metabolizing form of UDP-glucuronyltransferase in liver microsomes from Gunn rats is thus a diminished affinity for UDP-glucuronic acid as compared with enzyme from normal, Wistar rats (Table 1). Activity at V_{max} and the values of the Michaelis constant for p-nitrophenol, K_{PNP}, are identical in rats of the Gunn and Wistar strains.
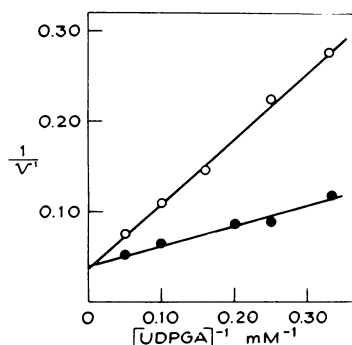

FIG. 2. Determination of activity at V_{max} of UDP-glucuronyltransferase, assayed in the forward direction, in microsomes from Gunn rats (O) and normal, Wistar rats (●). Initial rates of activity were determined as in Fig. 1 and *Materials and Methods* as a function of variable concentrations of p-nitrophenol at several different fixed concentrations of UDP-glucuronic acid. The reciprocals of the rates at infinite concentrations of p-nitrophenol (1/v^1) are plotted as a function of the reciprocals of each fixed concentration of UDP-glucuronic acid. The intercept on the ordinate is 1/V_{max} of the forward reaction. Units of activity are nmol of p-nitrophenol glucuronidated per min/mg of protein.

Table 1. Kinetic parameters of UDP-glucuronyltransferase for assays of the foward reaction

Source of liver microsomes	Kinetic parameter		
	V_{max}	K_{UDPGA}	K_{PNP}
Gunn rat	21.2	15.0	0.40
Wistar rat	21.2	4.0	0.33

Activities at V_{max} were determined as in Fig. 2. The values of K_{UDPGA} were determined graphically from a replot of the slopes and intercepts of the data in Fig. 1 versus the different fixed concentrations of p-nitrophenol (15). The values of K_{PNP} were determined graphically in a similar manner except that the primary data were plotted as a function of the concentration of p-nitrophenol. Units of activity are nmol of p-nitrophenol glucuronidated per min/mg of protein; units of the K terms are mM. See *text* for physical meaning of K_{UDPGA} and K_{PNP}.

Interaction of UDP-glucuronyltransferase with the UDP portion of UDP-glucuronic acid was studied by determining the kinetic parameters of the reverse reaction catalyzed by this enzyme. The rate of the reverse reaction was decreased in liver microsomes from Gunn rats, as compared with those from Wistar rats. The basis of this decrease of rate is decreased affinity of UDP-glucuronyltransferase for UDP (Table 2). The differences between activities at V_{max} and K_{PNPGA} for Gunn and Wistar rats (Table 2) are not significant.

The conformation of UDP-glucuronic acid in solution is unknown, as is the nature of the contacts between this compound and UDP-glucuronyltransferase. Nevertheless, the binding of UDP-glucuronic acid to UDP-glucuronyltransferase can be considered to depend on interactions between the enzyme and regions of the UDP and glucuronic acid moieties. The defective binding of substrate to the enzyme of Gunn rats could reflect abnormalities in enzyme–UDP or enzyme–glucuronic acid interactions, or both. The data in Tables 1 and 2 indicate abnormal binding of the UDP portion of UDP-glucuronic acid to the enzyme. An estimate of the energy associated with enzyme–glucuronic acid interactions can be obtained from the kinetic data since values of $\Delta G°$ of the binding of glucuronic acid to UDP-glucuronyltransferase can be calculated from the K terms. Thus, $\Delta G°$ of the contribution to binding of glucuronic acid may be approximated as the difference between $\Delta G°$ of binding for UDP-glucuronate and $\Delta G°$ of binding for UDP. This difference is 2.4 kcal/mol (10 kJ/mol) for Wistar rats and 2.7 kcal/mol (11 kJ/mol) for Gunn rats. These results suggest that the defect in Gunn rats is due primarily to abnormalities in interactions between the enzyme and the UDP portion of UDP-glucuronic acid. Evidence bearing on this question also was obtained from studies of inhibition by UDP of the rate of glucuronidation of p-nitrophenol.

Table 2. Kinetic parameters of UDP-glucuronyltransferase for assays of the reverse reaction

Source of liver microsomes	Kinetic parameter		
	V_{max}	K_{UDP}	K_{PNPGA}
Gunn rat	0.62	0.18	12.5
Wistar rat	0.88	0.06	10.0

Kinetic parameters were determined by graphical methods as in Table 1. Units are the same as in Table 1, except that V_{max} is in terms of p-nitrophenol produced.

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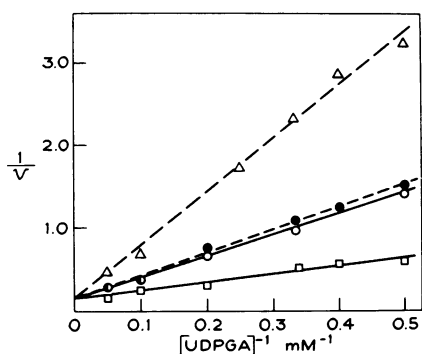
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FIG. 3. Effect of diethylnitrosamine on the activity of UDP-glucuronyltransferase. Initial rates of activity of UDP-glucuronyltransferase were determined as in *Materials and Methods*. Microsomes were from Gunn rats (Δ, ●) or normal, Wistar rats (O, □). The concentration of p-nitrophenol was 0.4 mM in all assays. The concentrations of UDP-glucuronic acid are indicated in the figure. The concentration of diethylnitrosamine, when added, (●, □) was 10 mM. The data are plotted in double reciprocal form as a function of the concentration of UDP-glucuronic acid. Units of activity are nmol of p-nitrophenol glucuronidated per min/mg of protein.
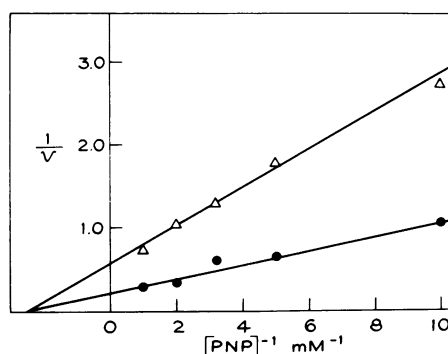
FIG. 4. Effect of diethylnitrosamine on the activity of UDP-glucuronyltransferase in microsomes from Gunn rats. Initial rates of activity were determined as in *Materials and Methods* using 1 mM UDP-glucuronic acid and the indicated concentrations of p-nitrophenol (PNP). No added diethylnitrosamine (Δ); 10 mM diethylnitrosamine (●). Data are plotted in double reciprocal form. Units of activity are nmol of p-nitrophenol glucuronidated per min/mg of protein.

The extent of UDP-induced inhibition of the rate of the forward reaction of UDP-glucuronyltransferase depends on the relative affinities of the enzyme for UDP-glucuronic acid and UDP. A change in the affinity of the enzyme for the UDP moiety should affect equally the affinity of the enzyme for UDP and UDP-glucuronic acid, and would not be expected to alter the extent of end-product inhibition by UDP. On the other hand, a change in the affinity of the enzyme for the glucuronic acid moiety will have a differential effect on the affinities of the enzyme for UDP and UDP-glucuronic acid, and thus should alter the extent of product inhibition by UDP. The effects of UDP on rates of glucuronidation by UDP-glucuronyltransferase of Gunn and Wistar rats were compared for this reason. The extent of inhibition of UDP-glucuronyltransferase by UDP was the same with enzyme from Gunn or Wistar rats (Table 3). These data hence support the conclusion that the defect in the Gunn rat is in the interactions between UDP-glucuronyltransferase and the UDP portion of UDP-glucuronic acid.

Effect of diethylnitrosamine on the kinetic parameters of UDP-glucuronyltransferase

Addition of diethylnitrosamine to microsomes increases the abnormally low activity of UDP-glucuronyltransferase of Gunn rats to nearly normal levels, when the enzyme is assayed with several different aglycones (4, 17). Complete bisubstrate kinetic analyses were not carried out, but the data in Figs. 3 and 4 indicate that treatment with diethylni-

Table 3. UDP inhibition of the glucuronidation rate of p-nitrophenol

Source of UDP-glucuronyltransferase	UDP added to assay	Activity
Gunn rat	—	0.22
	+	0.10
Wistar rat	—	0.40
	+	0.18

Initial rates of activity of UDP-glucuronyltransferase were determined as in *Materials and Methods* using 0.4 mM p-nitrophenol and 2 mM UDP-glucuronic acid as substrates. When added, the concentration of UDP was 2.0 mM. Activities are nmol of p-nitrophenol glucuronidated per min/mg of protein.

trosamine increases the activity of the p-nitrophenol-metabolizing form of UDP-glucuronyltransferase in liver microsomes from Gunn rats by reversing the genetically determined defect. Treatment with diethylnitrosamine increases the apparent affinity of UDP-glucuronyltransferase for UDP-glucuronic acid. Diethylnitrosamine does not alter activity at infinite concentrations of UDP-glucuronic acid (Fig. 3), nor does it modify the apparent K_{PNP} (Fig. 4). The data in Fig. 3 also indicate that rates of glucuronidation are increased when UDP-glucuronyltransferase from Wistar rats is assayed in the presence of diethylnitrosamine. The activity of the enzyme from Wistar rats is slightly greater in the presence of diethylnitrosamine than activity of diethylnitrosamine-treated microsomes from Gunn rats.

Treatment with diethylnitrosamine stimulates the rate of the reverse reaction catalyzed by UDP-glucuronyltransferase. Stimulation in this case also seems to result from an increase in the affinity of UDP-glucuronyltransferase for UDP (Fig. 5A and B). Treatment with diethylnitrosamine did not alter the extent of end-product inhibition by UDP of the rate of synthesis of p-nitrophenylglucuronic acid.

Effect of UDP-N-acetylglucosamine on the activity of UDP-glucuronyltransferase in Gunn rats

UDP-N-acetylglucosamine increases the activity of UDP-glucuronyltransferase (7, 18, 19) by enhancing the apparent affinity of the enzyme for UDP-glucuronic acid (7). In view of the defect in the binding of this substrate to the enzyme from Gunn rats, it was of interest to determine the effect on activity of treatment with UDP-N-acetylglucosamine. The data in Table 4 indicate that the activity of the UDP-glucuronyltransferase from Gunn rats is stimulated by UDP-N-acetylglucosamine. There is, in addition, an additive effect of treatment with diethylnitrosamine and UDP-N-acetylglucosamine. Since the effect of UDP-N-acetylglucosamine is dependent on divalent metal ions, EDTA was omitted from the assays in Table 4. As a result, the activities in this table are higher than those in Table 3.

DISCUSSION

The abnormally slow rate of glucuronidation of p-nitrophenol catalyzed by UDP-glucuronyltransferase of Gunn rats results from a poor affinity of the enzyme for UDP-glucuronic acid. This defect of function is limited, however, in that UDP-glucuronic acid binds to the defective form of the
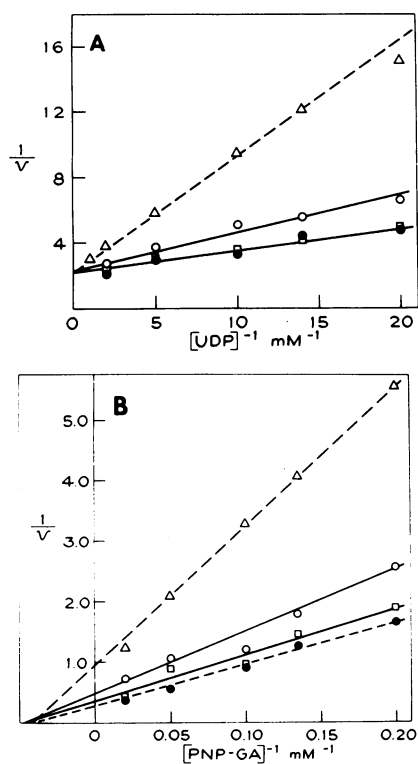
FIG. 5. Effect of diethylnitrosamine on rates of the reverse reaction catalyzed by UDP-glucuronyltransferase. Initial rates of the activity of UDP-glucuronyltransferase, assayed in the reverse direction, were determined as in *Materials and Methods*. Microsomes were from Gunn rats ($\triangle$, $\bullet$) or 'normal,' Wistar rats (O, $\square$). Activities were measured as a function of variable concentrations of UDP (A) at a fixed concentration of 10 mM p-nitrophenylglucuronic acid, or as a function of variable concentrations of p-nitrophenylglucuronic acid (PNP-GA) (B) at a fixed concentration of 3.0 mM UDP. When added, the concentration of diethylnitrosamine was 10 mM ($\bullet$, $\square$). Rates (nmol of p-nitrophenol produced per min/mg of protein) are plotted in double reciprocal form.

enzyme under conditions easily achieved *in vitro*. The p-nitrophenol-metabolizing form of UDP-glucuronyltransferase of Gunn rats can be regarded, therefore, as a minimally defective form of the enzyme. A critical unanswered question is the relationship between the abnormality of the p-nitrophenol glucuronidation reaction in Gunn rats and the more extensive abnormalities in the rates of glucuronidation of aglycones such as o-aminophenol, o-aminobenzoic acid, and bilirubin.

As pointed out previously, there is kinetic evidence that

Table 4. UDP-N-acetylglucosamine stimulation of the activity of UDP-glucuronyltransferase in microsomes from Gunn rats

Additions to assay	Activity
None	0.46
UDP-N-acetylglucosamine (4 mM)	1.40
Diethylnitrosamine (10 mM)	1.12
UDP-N-acetylglucosamine (4 mM) + diethylnitrosamine (10 mM)	3.30

Initial rates of activity of UDP-glucuronyltransferase were measured as in *Materials and Methods* except that EDTA was omitted from all assays. The concentration of p-nitrophenol was 0.4 mM and UDP-glucuronic acid was 1 mM in all assays. Activities are nmol of p-nitrophenol glucuronidated per min/mg of protein.

each of the above compounds is glucuronidated at a distinct active site (6–8). In addition, variability of the time of appearance of the different activities of UDP-glucuronyltransferase during fetal development and shortly after birth (3), as well as comparisons of the different activities in various species (2, 3), supports the idea of multiple, aglycone-specific forms of UDP-glucuronyltransferase. There is only a small number of genetic mechanisms that can account for defects in the function of multiple proteins. Abnormalities of regulator or operator function do not seem to apply to the Gunn rat because of the differences in the extent of the defect of rates of glucuronidation of various aglycones. Also, since treatment with diethylnitrosamine increases the rates of glucuronidation of o-aminophenol, o-aminobenzoic acid, and p-nitrophenol to near normal levels, it is likely that normal amounts of defective forms of UDP-glucuronyltransferases are synthesized by Gunn rats. A hypothesis that accounts for the data in Gunn rats and predicts the defective function of multiple enzymes is that different UDP-glucuronyltransferases contain a common UDP-glucuronic acid binding subunit. A single mutation in such a common subunit would lead to defective function of many enzymes. Interactions of this subunit with different aglycone-binding subunits could modify the properties of the binding site of UDP-glucuronic acid, leading thereby to variability in the extent of the defect of the glucuronidation of different aglycones. These ideas can be tested by examination of the kinetic parameters of UDP-glucuronyltransferase assayed with aglycones other than p-nitrophenol.

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