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DIFFERENTIATION OF HOMOLOGOUS FORMS OF HEPATIC MICROSOMAL UDP-GLUCURONYLTRANSFERASE*

I. EVIDENCE FOR THE GLUCURONIDATION OF *o*-AMINOPHENOL AND *p*-NITROPHENOL BY SEPARATE ENZYMES

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SUMMARY

Using kinetic methods it has been possible to show that there are separate forms of UDPglucuronyltransferase (EC 2.4.1.17) for the synthesis of *p*-nitrophenylglucuronide and *o*-aminophenylglucuronide. Thus, *p*-nitrophenylglucuronide, which is a product inhibitor of its own synthesis, had no effect of the rate of synthesis of *o*-aminophenylglucuronide; and 4-methylumbelliferylglucuronide and phenylglucuronide were competitive inhibitors of the glucuronidation of *o*-aminophenol but had no effect on that of *p*-nitrophenol. Since previous measurements of the rate of glucuronide synthesis as a function of the concentration of UDPglucuronic acid demonstrated that these aglycones did not share a common UDPglucuronic acid binding site, it was concluded that each phenol was metabolized by a separate form of UDPglucuronyltransferase.

In order to compare the properties of the two forms of UDPglucuronyltransferases the effect of sulfhydryl group reagents on activities was studied making use of the fact that the *p*-nitrophenol form of the enzyme is known to have at least three distinctly different sulfhydryl groups. The *o*-aminophenol form also contained three such groups with nearly identical reactivities as the *p*-nitrophenol form, indicating close structural similarities between these enzymes. On the other hand, the sulfhydryl groups of the two forms of UDPglucuronyltransferase could be distinguished by their rates of reaction with mersalyl.

INTRODUCTION

An interesting characteristic of some microsomal enzymes is their catalysis of a homologous reaction with a series of differing substrates, as for example in hydroxylating, acylating, and glucuronidating reactions and the synthesis of glycoproteins.

For none of these systems is it clear how many different, substrate-specific enzymes exist, or in fact whether for each type of reaction there is only a single enzyme with broad specificity. For example, relative rates of glucuronidation reactions with different substrates are not constant during fetal development, or adult life[1], and rates of hydroxylation reactions with different substrates are influenced selectively by administration of drugs[2–4]. These data suggest a multiplicity of enzymes for glucuronidation and hydroxylation reactions. This conclusion is weakened, however, by the observations that *in vitro* alterations of enzyme–phospholipid interactions in the microsomal membrane modify independently either the phosphotransferase or phosphohydrolase activities of glucose-6-phosphatase (EC 3.1.3.9)[5], and that these activities also can vary independently of each other *in vivo*[6]. The apparent multiplicity of some microsomal enzyme systems may be related, therefore, to variability in lipid protein relationships in the microsome. This cannot be established, however, without independent information on the number and substrate specificities for each family of enzymes.

Our interest in the problem of the multiplicity of microsomal enzymes has focussed on UDPglucuronyltransferase (EC 2.4.1.17). Although O- and N-glucuronidating activities reportedly can be separated[7], it is not known how many O-glucuronidating forms there are. Because of the difficulty in separating these enzymes from the microsomal membranes, and due to the fact that available methods for "solubilizing" membrane bound enzymes are certain to alter their properties[5,8,9], kinetic methods would seem to be the most useful technique for distinguishing different forms of UDPglucuronyltransferase. This idea has been reinforced by the resolution of some of the uncertainties in the function of glucose-6-phosphatase using kinetic methods[10–12]. Furthermore, studies in this laboratory suggest that the failure of previous kinetic studies to clarify some of the uncertainties of the multiplicity of UDPglucuronyltransferases resulted from poor experimental design[13,14]. In addition to kinetic approaches the problem of multiplicity can be investigated by taking advantage of the fact that there are three sulfhydryl groups important for the activity of UDPglucuronyltransferase assayed with *p*-nitrophenol as aglycone[15]. Thus, properties of the enzyme other than its kinetic parameters can be utilized for discriminating between possible multiple forms of UDPglucuronyltransferases.

MATERIALS AND METHODS

UDPglucuronic acid (ammonium salt), saccharic acid-1,4-lactone, *p*-nitrophenol, *o*-aminophenol and *p*-nitrophenylglucuronide were purchased from Sigma Chemical Co.; 4-methylumbelliferyl-, phenyl-, and *o*-aminophenylglucuronides were obtained from Koch-Light Laboratories, Colnbrook, England; *o*-amino[^{14}C]phenol was purchased from the Radiochemical Division of Mallinckrodt.

Livers were obtained from retired male breeder guinea pigs and microsomes isolated as described previously[14].

Enzyme assays

When the influence of sulfhydryl group reagents on the activity of UDPglucuronyltransferase was studied with *p*-nitrophenol as aglycone, assays were carried out at 37 °C in 50 mM phosphate buffer (pH 7.1); with *o*-aminophenol assays

were in 50 mM phosphate buffer (pH 7.6). The concentration of aglycones was 0.2 mM and UDPglucuronic acid 5.0 mM. Activities were based on initial rates of disappearance of *p*-nitrophenol, or the appearance of *o*-aminophenylglucuronide[14]. The extinction coefficient for the diazotized *o*-aminophenylglucuronide after coupling with *N*-(1-naphthyl)ethylenediamine (555 nm) was determined daily. For kinetic experiments activities with both aglycones were measured in 50 mM phosphate buffer (pH 7.6). The concentrations of substrates in kinetic experiments are indicated in the figures and table.

When the effects of glucuronides as product inhibitors were investigated with either *p*-nitrophenol or *o*-aminophenol as aglycone, 5.0 mM saccharic acid-1,4-lactone was added to each assay tube[16]. The effect of *o*-aminophenylglucuronide as a product inhibitor of its own synthesis was examined using a radiochemical assay with *o*-amino[^{14}C]phenol as aglycone. Assays were conducted as with unlabelled *o*-aminophenol, except that after stopping each reaction, unreacted *o*-amino[^{14}C]-phenol was removed by repeated extractions with diethyl ether which effected a complete separation of substrate and product. The *o*-amino[^{14}C]phenyl glucuronide synthesized, which remained in the aqueous phase, was measured by liquid scintillation counting. Proteins were measured with the biuret method[17].

RESULTS

The properties of the aglycone and UDPglucuronic acid binding sites of UDPglucuronyltransferase

Data reported in the literature, which indicate that *p*-nitrophenol is a competitive inhibitor of the synthesis of *o*-aminophenylglucuronide, have been interpreted as signifying that *p*-nitro- and *o*-aminophenols are glucuronidated by the same enzyme[18]. Data in Table I are at first glance compatible with this conclusion also in that they show mutual inhibition by *o*-amino- and *p*-nitrophenols of the rate of synthesis of their glucuronides. We would stress, however, that these data and those referred to above[18] cannot exclude the possibilities that *p*-nitrophenol forms a dead-end complex with a form of UDPglucuronyltransferase catalyzing only the synthesis of *o*-aminophenylglucuronide and *vice versa*, or that there are separate binding sites for *o*-amino- and *p*-nitrophenol but a single site for UDPglucuronic acid. The problem, of separate aglycone sites, can be studied directly, in principle, by comparing K_p-

TABLE I

SYNTHESIS OF GLUCURONIDES FROM *o*-AMINOPHENOL AND *p*-NITROPHENOL

UDPglucuronyltransferase was assayed in 50 mM phosphate buffer (pH 7.6) as in Materials and Methods with 10 mM UDPglucuronic acid, and either 0.10 mM *p*-nitrophenol, 0.04 mM *o*-aminophenol, or both as glucuronyl acceptors.

Aglycone	*Glucuronide synthesized (nmoles per min per mg protein)*
p-Nitrophenol	13.9
o-Aminophenol	1.75
p-Nitrophenol + *o*-aminophenol	6.37

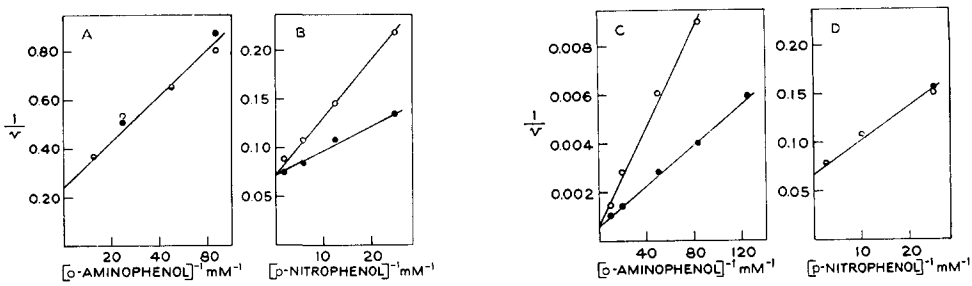


Fig. 1. Product inhibition of UDPglucuronyltransferase by *p*-nitrophenylglucuronide and *o*-aminophenylglucuronide. Activities were measured as in Materials and Methods, at pH 7.6; 5.0 mM saccharic-1,4-lactone was added to each tube in order to inhibit β-glucuronidase. The concentration of UDPglucuronic acid was 5.0 mM in all assays. A. Reciprocal rates of glucuronidation of *o*-aminophenol, expressed as absorbance change per min per mg microsomal protein, in the presence (○) and absence (●) of 10 mM *p*-nitrophenylglucuronide. B. Reciprocal rates of glucuronidation of *p*-nitrophenol in presence (○) and absence (●) of 10 mM *p*-nitrophenylglucuronide. C. Reciprocal rates of glucuronidation of *o*-aminophenol, expressed as cpm per mg microsomal protein per min, in the presence (○) and absence (●) of 10 mM *o*-aminophenylglucuronide. D. Same as (B) except 10 mM *o*-aminophenylglucuronide was substituted for *p*-nitrophenylglucuronide.

nitrophenol for the synthesis of *p*-nitrophenylglucuronide with K_i for *p*-nitrophenol as an inhibitor of the synthesis of *o*-aminophenylglucuronide. Alternatively, the products, *o*-aminophenyl- and *p*-nitrophenylglucuronides, could be used in a similar way. Because, as we have pointed out previously[14], phenols at moderate concentrations have non-specific activating effects on UDPglucuronyltransferase, we elected to study the properties of the aglycone binding site using alternate products as inhibitors rather than alternate substrates. The results of the product inhibition studies provided a surprisingly direct and easily arrived at answer as to whether *o*-amino- and *p*-nitrophenols are glucuronidated at identical active sites.

As is evident in Fig. 1A, 10 mM *p*-nitrophenylglucuronide did not inhibit the synthesis of *o*-aminophenylglucuronide at concentrations of *o*-aminophenol as low as 0.012 mM, which is considerably below that needed to half saturate the enzyme at 5.0 mM UDPglucuronic acid. The data in Fig. 1B indicate, on the other hand, that

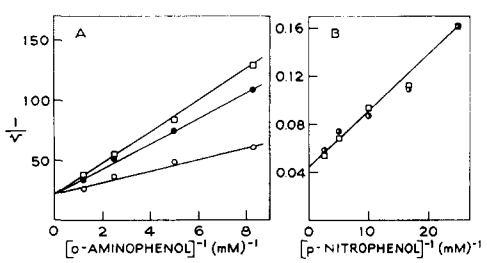


Fig. 2. The effect of 10 mM phenylglucuronide and 10 mM 4-methylumbelliferylglucuronide on the glucuronidation of *o*-amino- (A) and *p*-nitrophenol (B). Activities were measured as in Fig. 1 except that in (B) the concentration of UDPglucuronic acid was 10.0 mM. In (A) *v* is expressed as the absorbance change per min per mg protein and in (B) as nmoles *p*-nitrophenol consumed per min per mg protein. No inhibitors (○); *plus* phenylglucuronide (●); or *plus* 4-methylumbelliferylglucuronide (□). The symbol (◑) indicates coincidence of points for no added inhibitor or in the presence of phenylglucuronide.

10 mM *p*-nitrophenylglucuronide was a competitive inhibitor of its own synthesis. Similarly, 10 mM *o*-aminophenylglucuronide was a competitive inhibitor of its own synthesis (Fig. 1C), but was without effect on the rate of synthesis of *p*-nitrophenylglucuronide (Fig. 1D). Thus, there are at least two separate sites for the glucuronidation of *p*-nitrophenol and *o*-aminophenol. Further evidence for the separateness of these sites came from experiments with phenylglucuronide and 4-methylumbelliferylglucuronide, both of which were competitive inhibitors of the synthesis of *o*-aminophenylglucuronide but were without effect on either the rate of synthesis of *p*-nitrophenylglucuronide (Fig. 2), or the rate of UDP-dependent hydrolysis (reverse reaction).

While the binding sites for *o*-amino- and *p*-nitrophenol are distinct, it is still possible that the two aglycones share a common UDPglucuronic acid binding site. We therefore considered separately the problem of the dependence of the rates of synthesis of *p*-nitro- and *o*-aminophenylglucuronides as a function of the concentration of UDPglucuronic acid.

As with *p*-nitrophenol or *o*-aminobenzoate[19], double reciprocal plots for the rate of synthesis of *o*-aminophenylglucuronide as a function of the concentration of UDPglucuronic acid were non-linear. Since this non-linearity could not have arisen from artifacts of the assay[19], it was considered to be a real property of the enzyme catalyzing the synthesis of *o*-aminophenylglucuronide. The rate data, plotted in the form of the Hill equation, for assays with *p*-nitro- or *o*-aminophenols as aglycones, have limiting slopes of approx. 1 at high concentrations of UDPglucuronic acid and slopes of less than 1 at concentrations of UDPglucuronic acid less than 2.5 mM (Fig. 3); but

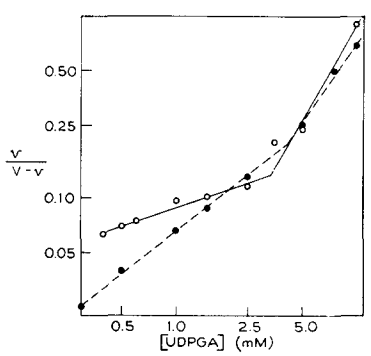


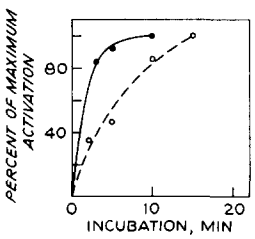
Fig. 3. Comparison of the rate of synthesis of *o*-aminophenylglucuronide (○) and *p*-nitrophenylglucuronide (●) as a function of the concentration of UDPglucuronic acid (UDPGA). Enzyme activities were determined as in Materials and Methods with 0.2 mM *p*-nitrophenol or 0.2 mM *o*-aminophenol and the indicated concentrations of UDPglucuronic acid. Data are plotted according to the Hill equation.

the slopes at low concentrations of UDPglucuronic acid differed for assays with *o*-amino- or *p*-nitrophenols as aglycone, suggesting that the glucuronidation of these compounds does not share a common UDPglucuronic acid site. Thus, the evidence on the binding of aglycones and UDPglucuronic acid indicates that *o*-amino- and *p*-nitrophenols are metabolized by separate forms of UDPglucuronyltransferase.

Effect of mersalyl on the activity of UDPglucuronyltransferase with o-aminophenol and p-nitrophenol as aglycones

With *p*-nitrophenol as glucuronyl acceptor, UDPglucuronyltransferase has three distinct sulfhydryl groups[15]. One of these reacts with *N*-ethylmaleimide, producing slight activation, and has been designated Type 1. The Type 2 sulfhydryl group reacts with relatively low concentrations of organic mercurials and produces a large increase in activity. The Type 3 sulfhydryl group is titrated by relatively large concentrations of organic mercurial leading to a reversible deactivation of the activated enzyme. Since the kinetic data indicate that *o*-amino- and *p*-nitrophenols are metabolized by different forms of UDPglucuronyltransferase, treatment of microsomes with sulfhydryl group reagents is likely to have different effects on the rates of synthesis of *o*-aminophenylglucuronide and *p*-nitrophenylglucuronide.

Comparison of the effect of *N*-ethylmaleimide and mersalyl on the activity of UDPglucuronyltransferase assayed with *o*-aminophenol or *p*-nitrophenol as aglycones, revealed, however, that plots of concentration of sulfhydryl group reagent *versus* enzyme activity were nearly identical with either *o*-aminophenol or *p*-nitrophenol as aglycones. The only significant difference observed for assays with these two substrates was the greater extent of mersalyl-induced activation of *p*-nitrophenol glucuronidation as compared with that for *o*-aminophenol. On the other hand, studies of the rate of mersalyl-induced activation of glucuronidation of *o*-amino- and *p*-nitrophenols demonstrated differences between the two forms of UDPglucuronyltransferase. With *p*-nitrophenol as aglycone maximal activation is achieved after



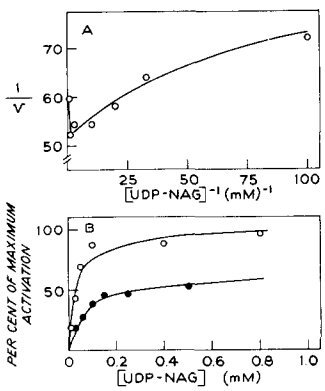


Fig. 4. Time course of mersalyl-induced activation of UDPglucuronyltransferase assayed with *p*-nitrophenol (○) or *o*-aminophenol (●) as glucuronyl acceptors. Microsomes in 50 mM Tris buffer (pH 7.6) at a protein concentration of 10 mg/ml were brought to 23 °C. Mersalyl to a final concentration of 0.5 mM was added and aliquots were removed for assay of UDPglucuronyltransferase as in Materials and Methods and in Fig. 1 at the times indicated. The data have been normalized by plotting the percent of maximum activation at each time point.

Fig. 5. Effect of the concentration of UDP-*N*-acetylglucosamine on the rate of synthesis of *o*-aminophenylglucuronic acid. The rate of synthesis of *o*-aminophenylglucuronic acid was determined as in Materials and Methods with 0.2 mM *o*-aminophenol, 0.5 mM UDPglucuronic acid, and the indicated concentrations of UDP-*N*-acetylglucosamine; *v* is expressed as the change in absorbance per min per mg microsomal protein. A. Double reciprocal plots of initial rate data. B. The percent of maximum activation of glucuronidation for different concentrations of UDP-*N*-acetylglucosamine (UDP-NAG) with 0.2 mM *o*-aminophenol (○) or 0.2 mM *p*-nitrophenol (●) as glucuronyl acceptors.

15 min of treatment of microsomes with 0.5 mM mersalyl (titration of Type 2 sulfhydryl), but with *o*-aminophenol activation reached a peak level within 6 min (Fig. 4).

The rate of glucuronidation of o-aminophenol as a function of the concentration of UDP-N-acetylglucosamine

Addition of UDP-*N*-acetylglucosamine to assays increases the rate of synthesis of *o*-aminophenylglucuronide as well as *p*-nitrophenylglucuronide[19]. It was possible, however, to distinguish between the enzymes responsible for the glucuronidation of *o*-aminophenol and *p*-nitrophenol by comparison of the effects of UDP-*N*-acetylglucosamine on each reaction. Thus, as shown in Fig. 5A the non-linearity in plots of $1/v$ *versus* 1/[UDP-*N*-acetylglucosamine] is less extensive with *o*-aminophenol as aglycone as compared to *p*-nitrophenol[19]. This is reflected also in the fact that less UDP-*N*-acetylglucosamine is required for half maximal activation of the *o*-aminophenol reaction than with *p*-nitrophenol (Fig. 5B). Additionally, concentrations of UDP-*N*-acetylglucosamine greater than 5 mM inhibit the glucuronidation of *o*-aminophenol but activate the rate of synthesis of *p*-nitrophenylglucuronide (Fig. 5A and ref. 19).

DISCUSSION

As shown in this paper, *p*-nitrophenol and *o*-aminophenol are glucuronidated at separate binding sites, and the glucuronidation of these phenols does not share a common UDPglucuronic acid binding site. In addition, the binding properties of the effector, UDP-*N*-acetylglucosamine, are different when the enzyme is assayed with *o*-amino- or *p*-nitrophenols; and the properties of the sulfhydryl groups which influence the kinetic parameters of UDPglucuronyltransferase are different for the synthesis of *o*-aminophenyl- and *p*-nitrophenylglucuronides.

Equally important as the demonstration of more than one form of UDPglucuronyltransferase, the data cited indicate the usefulness of the method as a general approach to the problem of delineating more completely the number of microsomal UDPglucuronyltransferases and for studying the chemical basis for substrate specificity. The easily discernible differences in the properties of *p*-nitrophenylglucuronide as a product inhibitor of its own synthesis, but not of *o*-aminophenylglucuronide, and the differential rates of reactivity of the Type 2 sulfhydryl in the *o*-amino- and *p*-nitrophenol forms of UDPglucuronyltransferase should allow for deciding which of these two enzymes is responsible for the glucuronidation of a variety of phenolic substrates. The more general problem of the glucuronidation of bilirubin, other carboxyl aglycones and phenols which may be metabolized by enzymes other than the two delineated above can be attacked in a similar manner. Although the chemical basis is not completely clear, we would stress the advantage of using alternate products as inhibitors rather than alternate substrates for studies of the aglycone binding sites because of the greater specificity of the former compounds.

As pointed out in Introduction, the regulatory properties of UDPglucuronyltransferase cannot be elucidated without prior knowledge of the number of such enzymes. Other related problems which depend for their clarification on exact knowledge of the number and specificity of UDPglucuronyltransferases are the significance of the complex pattern for the development of different glucuronyltransferase activi-

ties in fetal and new-born animals, and the nature of the defect in the Gunn rat, in which there is a genetically determined abnormality in the rate of synthesis of some but perhaps not all glucuronides[1,20].

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