Effects of Metals on the Properties of Hepatic Microsomal Uridine Diphosphate Glucuronyltransferase†

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ABSTRACT: Divalent metal ions have two important effects on the properties of UDP-glucuronyltransferases. They alter the properties of the active sites of these enzymes and modify the effect of UDP-*N*-acetylglucosamine on their activities. The metal ion responsible for effects on the active site is different from the metal ion which modifies the properties of the allosteric site (UDP-*N*-acetylglucosamine site); that is, there are at least two metal binding sites in some forms of UDP-glucuronyltransferase. The effects of metal ions also differ, according to the compound used as glucuronyl acceptor. By studying the effects of metal ions on the rates of synthesis of *p*-nitrophenyl, *o*-aminobenzoyl, and *o*-aminophenyl glucuronides, three types of UDP-glucuronyltransferase reactions can be distinguished. In the type I reaction, for which *p*-nitrophenol serves as the prototype aglycone, divalent metal ions increase activity at V_{max}, and are essential for stimulation by UDP-*N*-acetylglucosamine. In contrast to the type I reaction, divalent metal ions are not essential for stimulation of UDP-glucuronyltransferase by UDP-*N*-acetylglucosamine with *o*-aminophenol as aglycone (type I_a reaction). Divalent metal ions, however, increase activity at V_{max} for the type Ia reaction. In the type II reaction, for which *o*-aminobenzoate is the prototype aglycone, metal ions decrease the concentrations of UDP-glucuronic acid needed for half-maximal rates of glucuronidation and are essential for stimulation by UDP-*N*-acetylglucosamine. Metal ions do not increase activity at V_{max} for the type II reaction. Interactions between metal ions and UDP-*N*-acetylglucosamine seem to be essential components of the regulatory apparatus of UDP-glucuronyltransferase catalyzed reactions since these interactions ensure rapid rates of glucuronidation under conditions *in vivo*.

Hepatic microsomal UDP-glucuronyltransferases (EC 2.4.1.17) appear to constitute a family of closely related enzymes responsible for the catalysis of a number of glucuronidation reactions. One of the interesting characteristics of these reactions is that their kinetic properties are modified by a variety of treatments (Vessey and Zakim, 1971, 1972a; Zakim and Vessey, 1972; Wisnes, 1972). Of these, UDP-*N*-acetylglucosamine, a naturally occurring metabolite, increases the rate of glucuronidation of a variety of aglycones by enhancing the affinity of UDP-glucuronyltransferases for UDP-glucuronic acid (Vessey *et al.*, 1973a,b; Zakim *et al.*, 1973a). Since the concentration of UDP-glucuronic acid in the intact liver is low relative to the concentration required *in vitro* for half-maximal rates of activity of UDP-glucuronyltransferase ($K_{0.5}$) (Vessey and Zakim, 1972b; Zhivkov, 1970), regulation of glucuronidation reactions by UDP-*N*-acetylglucosamine could be critical for the efficient function of these enzymes *in vivo*. Other naturally occurring agents which could influence the $K_{0.5}$ for UDP-glucuronic acid, or the turnover number of UDP-glucuronyltransferase, also would be important for the efficiency of detoxification *via* the synthesis of glucuronide derivatives. In this regard, several previous reports have suggested that Mg^{2+} and Ca^{2+} modulate the rate of synthesis of some glucuronides (Pogell and Leloir, 1961; Isselbacher *et al.*, 1962; Storey, 1965; Tomlinson and Yaffe, 1966; Mulder, 1971; Heirwegh *et al.*, 1972). There is wide variability in the reported effects of these metals, however, in that some authors have reported activation, whereas others have found inhibition. These discrepancies may result, in part, from the problems associated with metabolism of UDP-glucuronic acid in side pathways, failure to consider that there may be separate substrate-specific forms of UDP-glucuronyltransferase, and failure to measure initial rates of activity. In addition, the concentrations of metals which have been employed in prior studies are greater than those likely to be present *in vivo*. On the other hand, experiments in our laboratory, which were not primarily designed to investigate the effects of metals on the activities of UDP-glucuronyltransferases, indicated that divalent metal ions have important effects on the rate of synthesis of *p*-nitrophenyl glucuronide (Zakim *et al.*, 1973b). Of particular significance was that divalent metal ions were essential for activation of the rate of synthesis of this glucuronide by UDP-*N*-acetylglucosamine. Moreover, the effects of metal ions were evoked by concentrations likely to occur in intact liver. For these reasons the problem of the effect of metal ions on the activities of UDP-glucuronyltransferases has been investigated in detail.

As mentioned above, there appear to be several substrate specific forms of UDP-glucuronyltransferase, though it is uncertain how many such forms exist. Studies in this laboratory indicate, however, that there are at least separate aglycone sites for the conjugation of *p*-nitrophenol, *o*-aminophenol, and *o*-aminobenzoic acid (Zakim *et al.*, 1973a). Hence, in the present experiments the effects of metals on the rate of conjugation of each of these aglycones has been determined.

Materials and Methods

Microsomes from guinea pig livers were used as the source of UDP-glucuronyltransferase. Microsomes were isolated as described previously (Zakim and Vessey, 1973) after homogenization in either 0.25 M sucrose or 0.25 M sucrose contain-

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ing 0.005 M EDTA (pH 6.5). The microsomal pellets homogenized in sucrose–EDTA were resuspended and washed twice in 0.25 M sucrose. Microsomes were stored at −20°, but were discarded if not used within 4 weeks of the time of preparation.

UDP-glucuronic acid (ammonium salt) and UDP-*N*-acetylglucosamine were purchased from Sigma Chemical Co. Since preliminary experiments indicated that these UDP derivatives were contaminated with heavy metals, they were treated with an ion exchange resin prior to use. Chellex-100 (Bio-Rad) was washed extensively with H_2O and then 0.1 N HCl until the pH of the eluate fell to 3.0. The resin was then washed with 0.1 N NaOH until the pH of the eluate rose to approximately 7.6. One gram of the acid and base washed resin was added to 2 ml of an unbuffered solution containing either 50 mM UDP-glucuronic acid or 50 mM UDP-*N*-acetylglucosamine, and the mixture was allowed to stand at 0° for 1 hr. The resin was removed by centrifugation. The recovery of UDP derivatives in the supernatant was monitored by measuring the absorption at 260 nm, and was complete.

The activity of UDP-glucuronyltransferase was measured with *p*-nitrophenol, *o*-aminobenzoate, or *o*-aminophenol as aglycones. Assays with each aglycone were carried out in 50 mM Tris (pH 7.6) at 37°. The concentrations of UDP-glucuronic acid, UDP-*N*-acetylglucosamine, aglycones, metal ions, and EDTA are indicated in the legends of the figures and tables and in the text. The final concentration of protein in each assay was 1–2 mg/ml. For each assay, initial rates of activity were measured by removal of serial aliquots from the assay media and determination of the rate of disappearance of *p*-nitrophenol (Vessey and Zakim, 1971; Zakim and Vessey, 1973) or the rate of appearance of *o*-aminobenzoyl glucuronide (Vessey *et al.*, 1973b) and *o*-aminophenyl glucuronide (Zakim *et al.*, 1973a), using standard colorimetric techniques. With the assay conditions used, no blank reactions occurred, and there was no significant metabolism of substrates and products *via* alternate pathways. With *p*-nitrophenol and *o*-aminophenol as aglycones, activities are expressed as nmol of substrate conjugated per min per mg of protein. For assays with *o*-aminobenzoate as aglycone, activities represent the optical density change due to the synthesis of *o*-aminobenzoyl glucuronide per min per mg of protein. Protein was determined by the biuret method (Gornall *et al.*, 1949).

Results

Effects of Mn^{2+} on the Catalytic Properties of UDP-glucuronyltransferases. As reported previously, addition of Mn^{2+} to microsomes homogenized in sucrose–EDTA increases the rates of glucuronidation of *p*-nitrophenol (Zakim *et al.*, 1973b). A similar effect is observed for assays with *o*-aminobenzoic acid as aglycone (Figure 1). The kinetic mechanism of this rate enhancement is different, however, for these two substrates. With *p*-nitrophenol as aglycone, Mn^{2+} increases activity at V_{max} (Zakim *et al.*, 1973b). For assays with *o*-aminobenzoate, Mn^{2+} decreases the $K_{0.5}$ for UDP-glucuronic acid (Figure 1). The data in Figure 1A are anomalous in that they do not fit a Michaelis–Menten kinetic model. The reasons for the break in these double reciprocal plots is not established with certainty as yet but these data reflect a real property of the enzyme, not artifacts of the assay system. As discussed at length in a previous publication (Vessey *et al.*, 1973a), the deviations from Michaelis–Menten kinetics are thought to result from negative cooperativity in the binding of substrates to UDP-glucuronyltransferase.

Besides an effect on the $K_{0.5}$ for UDP-glucuronic acid or on the activity at V_{max} of UDP-glucuronyltransferase, divalent metal ions are essential for activation of UDP-glucuronyltransferase by UDP-*N*-acetylglucosamine. Thus, the positive allosteric effector UDP-*N*-acetylglucosamine does not enhance the rates of glucuronidation of *p*-nitrophenol (Zakim *et al.*, 1973b) or *o*-aminobenzoate (Table I) in microsomes treated previously with EDTA (Table I). After addition of Mn^{2+} to such microsomes, however, UDP-glucuronyltransferase is susceptible to the activating effect of UDP-*N*-acetylglucosamine.

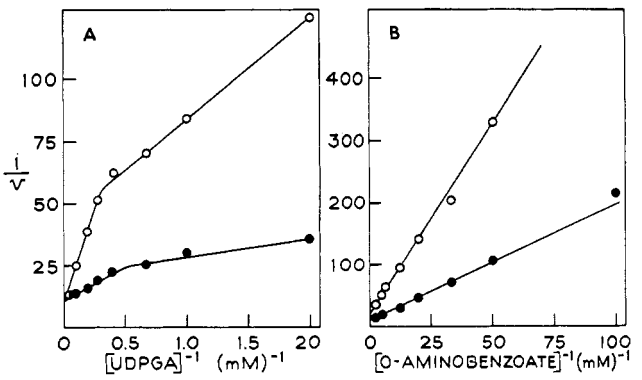

FIGURE 1: The influence of Mn^{2+} on the activities of UDP-glucuronyltransferase in microsomes from livers homogenized in sucrose–EDTA. Initial rates of activity of UDP-glucuronyltransferase were determined as in Methods with *o*-aminobenzoic acid as aglycone. In (A) the concentration of *o*-aminobenzoic acid was fixed at 0.2 mM, and in (B) the concentration of UDP-glucuronic acid was fixed at 1.0 mM. In all experiments the concentration of Mn^{2+} was either 1.0 mM (●), or no Mn^{2+} was added (○). Units of activity are the optical density change per min per mg of protein due to the synthesis of *o*-aminobenzoyl glucuronide.

Effects of Metal Ions Other Than Mn^{2+} on UDP-glucuronyltransferase. A large number of divalent metal ions influence the rate of glucuronidation of *p*-nitrophenol and *o*-aminobenzoate (Table II).

The effects of the metals on UDP-*N*-acetylglucosamine induced stimulation of UDP-glucuronyltransferase in Table II are similar, in general, to their effects on rates of glucuronidation in the absence of UDP-*N*-acetylglucosamine. Important exceptions are to be noted, however. Thus, Fe^{2+} does not alter the rate of synthesis of *p*-nitrophenyl glucuronide in the

TABLE I: Effect of Mn^{2+} on UDP-*N*-acetylglucosamine Stimulation of UDP-glucuronyltransferase.[a]

Additions	Activity
None	0.016
UDP-*N*-acetylglucosamine	0.017
$MnCl_2$	0.025
UDP-*N*-acetylglucosamine + $MnCl_2$	0.039

[a] Initial rates of activity of UDP-glucuronyltransferase were determined as in Methods using microsomes from livers homogenized in sucrose–EDTA as the source of the enzyme. The concentration of *o*-aminobenzoate in assay mixtures was 0.2 mM, and UDP-glucuronic acid 1.0 mM. When added, the concentration of UDP-*N*-acetylglucosamine was 2.0 mM. The concentration of $MnCl_2$, when present, was 0.05 mM. Units of activity are the optical density change per min per mg of protein due to the synthesis of *o*-aminobenzoyl glucuronide.

TABLE II: Effect of Metal Ions on the Activities of UDP-glucuronyltransferases.[a]

Added Metal	Act. with *o*-Aminobenzoic Acid as Aglycone		Act. with *p*-Nitrophenol as Aglycone	
	Act. in the Absence of UDP-*N*-acetylglucosamine	Act. in the Presence of UDP-*N*-acetylglucosamine	Act. in the Absence of UDP-*N*-acetylglucosamine	Act. in the Presence of UDP-*N*-acetylglucosamine
None	0.008	0.012	1.43	2.0
Mg^{2+}	0.018	0.036	2.42	12.1
Ca^{2+}	0.027	0.042	3.74	12.1
Mn^{2+}	0.030	0.052	4.40	15.4
Co^{2+}	0.023	0.028	4.18	11.0
Fe^{2+}	0.017	0.015	1.54	5.6
Zn^{2+}	0.010	0.005	0.33	1.1
Cu^{2+}	0.005	0.023	2.40	3.6

[a] Microsomes were from livers homogenized in 0.25 M sucrose–0.005 M EDTA. Initial rates of activity of UDP-glucuronyltransferases were determined as in Methods. The concentration of each aglycone was 0.2 mM, of each metal, 1 mM and UDP-glucuronic acid, 1.0 mM. When added, the concentration of UDP-*N*-acetylglucosamine was 2.0 mM. Units of activity are as in Table I and Methods.

TABLE III: Effect of EDTA on the Rate of Synthesis of *p*-Nitrophenyl Glucuronide.[a]

Concn of EDTA (mM)	Act. of Unstimulated Enzyme	Act. of UDP-*N*-acetylglucosamine Stimulated Enzyme
0	1.9	6.9
0.05	1.9	5.9
0.10	1.9	5.2
0.15	1.5	4.3
0.25	2.0	2.6
0.50	2.0	2.4
1.00	1.8	2.4
2.00		1.9

[a] Microsomes were from livers homogenized in 0.25 M sucrose, and the activity of UDP-glucuronyltransferase was determined as in Methods with 0.2 mM *p*-nitrophenol and 1.0 mM UDP-glucuronic acid as substrates. The concentrations of EDTA in each assay are indicated in the table. When added, the concentration of UDP-*N*-acetylglucosamine was 2.0 mM. Units of activity are nmol of *p*-nitrophenol metabolized per min per mg of protein.

absence of UDP-*N*-acetylglucosamine, but UDP-*N*-acetylglucosamine activates UDP-glucuronyltransferase if Fe^{2+} is added to EDTA-treated microsomes (Table II). In contrast to this result, Fe^{2+} increases the rate of synthesis of *o*-aminobenzoyl glucuronide in the absence of UDP-*N*-acetylglucosamine, but Fe^{2+} is without effect on UDP-*N*-acetylglucosamine induced activation of the *o*-aminobenzoyl metabolizing form of UDP-glucuronyltransferase. Cu^{2+} and Zn^{2+} also have differential effects on activity in the presence and absence of UDP-*N*-acetylglucosamine.

Evidence for at Least Two Separate Metal Binding Sites in UDP-glucuronyltransferases. The data for the effects of Fe^{2+}, Cu^{2+}, and Zn^{2+} on the activity of UDP-glucuronyltransferase assayed in the presence and absence of UDP-*N*-acetylglucosamine suggest that the effects of divalent metal ions on the properties of the active site and of the site at which UDP-*N*-acetylglucosamine binds are unrelated. More specifically, there appear to be two separate metal binding sites, one for the active site and the other for the UDP-*N*-acetylglucosamine site. This postulate was investigated further by studying the effects of EDTA on the rate of synthesis of glucuronides in microsomes prepared in 0.25 M sucrose, the reasoning for this approach being as follows. UDP-glucuronyltransferases in microsomes prepared in the absence of EDTA are activated by treatment with UDP-*N*-acetylglucosamine. Since this activation has an absolute dependence on metal ions with *o*-aminobenzoate and *p*-nitrophenol as aglycones (Table I), microsomes prepared in the absence of EDTA must contain some endogenous metals.

As expected, EDTA decreases the rate of synthesis of *p*-nitrophenyl glucuronide in the presence of UDP-*N*-acetylglucosamine (Table III). EDTA, however, has no effect on the rate of synthesis of *p*-nitrophenyl glucuronide by unstimulated UDP-glucuronyltransferase in microsomes prepared in 0.25 M sucrose. These microsomes, therefore, lack the metal which influences the properties of the active site. Nearly complete inhibition of UDP-*N*-acetylglucosamine activation is obtained at 0.25 mM EDTA. Thus, only small amounts of metals are required for the effect of UDP-*N*-acetylglucosamine. In fact, in the presence of concentrations of $MnCl_2$ as low as 2 μM UDP-*N*-acetylglucosamine activates EDTA-treated UDP-glucuronyltransferases (*p*-nitrophenol as aglycone). This concentration of $MnCl_2$ has no effect on the rate of glucuronidation in the absence of UDP-*N*-acetylglucosamine.

In contrast to results for the *p*-nitrophenol-metabolizing form of UDP-glucuronyltransferase, addition of EDTA to microsomes homogenized in sucrose inhibits the rate of synthesis of *o*-aminobenzoyl glucuronide in the presence or absence of UDP-*N*-acetylglucosamine (Table IV). The concentration of EDTA required to inhibit stimulation by UDP-*N*-acetylglucosamine is considerably smaller, however, than that needed to inhibit activity in the absence of UDP-*N*-acetylglucosamine. The *o*-aminobenzoate-metabolizing form of UDP-glucuronyltransferase therefore also contains at least two metal binding sites.

Metal Binding and Interactions with UDP-N-acetylglucosamine in Assays with p-Nitrophenol as Aglycone. Double reciprocal plots for the rate of glucuronidation of *p*-nitrophenol as a function of the concentration of Mn^{2+} are linear for concentrations of Mn^{2+} greater than 0.3 mM if the data are corrected for the fact that *p*-nitrophenol is glucuronidated in the absence of metals. Below 0.3 mM the corrected double reciprocal plots bend concave downward. The apparent $K_{Mn^{2+}}$ in assays utilizing *p*-nitrophenol as aglycone is about 0.8 mM. The concentration of UDP-glucuronic acid has no effect on this value.

Because metal ions and UDP-*N*-acetylglucosamine both modify the $K_{0.5}$ for UDP-glucuronic acid, it is important to determine whether these effectors interact. This problem cannot be studied in a direct manner, however, since UDP-glucuronyltransferase is bound firmly to the microsomal mem-

TABLE IV: Effect of EDTA on the Rate of Synthesis of *o*-Aminobenzoyl Glucuronide.[a]

Concn of EDTA (mM)	Act. of Un-stimulated Enzyme	Act. of UDP-*N*-acetylglucosamine Stimulated Enzyme	% Max. Inhibn of Un-stimulated Enzyme	% Max. Inhibn of UDP-*N*-acetyl-glucosamine Stimulation
0	0.0115	0.0389		
0.05	0.0101	0.0232	17.7	52.2
0.10	0.0087	0.0144	35	79.2
0.15	0.0072	0.0109	54	86.5
0.25	0.0036	0.0054	100	93.5
0.40	0.0036	0.0036	100	100
1.00	0.0036	0.0036	100	100

[a] Microsomes were from livers homogenized in 0.25 M sucrose. The activity of UDP-glucuronyltransferase was measured as in Methods with 0.2 mM *o*-aminobenzoate and 1.0 mM UDP-glucuronic acid as substrates. The concentrations of EDTA are indicated. When added, the concentration of UDP-*N*-acetylglucosamine was 2.0 mM. Units of activity are optical density change per min per mg of protein due to the formation of *o*-aminobenzoyl glucuronide. The per cent maximum inhibition was calculated by subtracting the activity in the presence of a given concentration of EDTA from that in the absence of EDTA and dividing this number by the difference in activities between untreated enzyme and enzyme assayed in the presence of 1.0 mM EDTA.

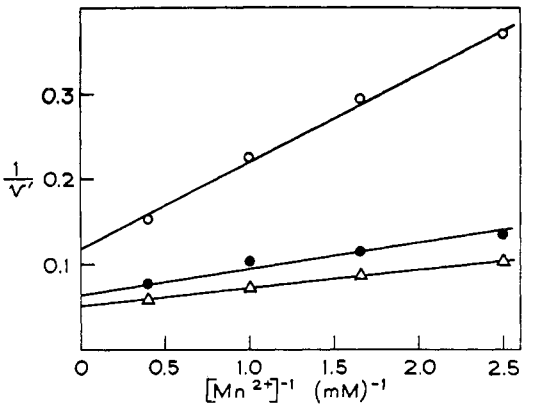

FIGURE 2: The rate of synthesis of *p*-nitrophenyl glucuronide as a function of the concentration of Mn^{2+} at variable fixed concentrations of UDP-*N*-acetylglucosamine. Microsomes from livers homogenized in sucrose–EDTA, as in Methods, were assayed for UDP-glucuronyltransferase activity as in Figure 1 in 0.2 mM *p*-nitrophenol, and 1.0 mM UDP-glucuronic acid. The concentrations of UDP-*N*-acetylglucosamine were 0 (○), 0.5 mM (●), or 2.5 mM (△); v' is the rate of synthesis of *p*-nitrophenyl glucuronide corrected for the rate of glucuronidation in the absence of Mn^{2+}. Units of activity are nmol of *p*-nitrophenol metabolized per min per mg of protein.

brane, and there are no currently useful methods for purifying an active form of the enzyme. In addition, the properties of this enzyme are sensitive to changes in its lipid environment (Zakim and Vessey, 1972; Vessey and Zakim, 1971). As a result, the properties of a soluble, purified form of the enzyme may not reflect those of the untreated or native form of the enzyme. Hence, direct binding studies are precluded at the present time. We, therefore, attempted to study the effect of variable concentrations of metals on the activity of UDP-glucuronyltransferase with *p*-nitrophenol as aglycone as a function of variable fixed concentrations of UDP-*N*-acetylglucosamine. This seemed reasonable, at least with *p*-nitrophenol as glucuronyl acceptor, since only a small amount of Mn^{2+} is needed to sensitize the enzyme to activation by UDP-*N*-acetylglucosamine, as compared with the concentration of Mn^{2+} needed to affect the activity at V_{max}.

The data in Figure 2 are double reciprocal plots of activity as a function of the concentration of Mn^{2+} in the presence or absence of UDP-*N*-acetylglucosamine. Two important points are illustrated by these data. First, UDP-*N*-acetylglucosamine increases the rate of glucuronidation of *p*-nitrophenol at infinite concentrations of Mn^{2+}. Second, and perhaps more important from the point of view of function *in vivo*, UDP-*N*-acetylglucosamine decreases the apparent K_m of UDP-glucuronyltransferase for Mn^{2+}.

Metal Binding and Interactions with UDP-N-acetylglucosamine for Assays with o-Aminobenzoate as Aglycone. In assays with *o*-aminobenzoate as aglycone, Mn^{2+} and UDP-*N*-acetylglucosamine both decrease the $K_{0.5}$ of UDP-glucuronic acid for UDP-glucuronyltransferase. Their effects on activity are additive only at low concentrations of each agent. UDP-*N*-acetylglucosamine does not increase the rate of synthesis of *o*-aminobenzoyl glucuronide at infinite concentrations of metals.

Because of the overlap in the concentrations of metals needed for direct effects at the active site and for expression of the UDP-*N*-acetylglucosamine effect, kinetic methods are not useful for investigating the effect of UDP-*N*-acetylglucosamine on the apparent $K_{Mn^{2+}}$ of UDP-glucuronyltransferase assayed with *o*-aminobenzoate. Interactions between the binding of metal ions and UDP-*N*-acetylglucosamine are apparent, however, in studies of the rate of synthesis of *o*-aminobenzoyl glucuronide as a function of variable concentrations of UDP-*N*-acetylglucosamine at several different fixed concentrations of metals. Increasing concentrations of Mn^{2+} decrease the apparent $K_{UDP\text{-}N\text{-}acetylglucosamine}$ of UDP-glucuronyltransferase for assays with *o*-aminobenzoate (Figure 3). Concentrations of UDP-*N*-acetylglucosamine less than those reported to occur *in vivo* are sufficient, in fact, to nearly saturate UDP-glucuronyltransferase in the presence of relatively small concentrations of divalent metal ions. The data in Figure 3 also reveal that at infinite concentrations of UDP-*N*-acetylglucosamine the corrected rates of glucuronidation of *o*-aminobenzoate are independent of the concentration of metal ions, though a small amount of metal is necessary (Table I).

Effects of Metal Ions on the Rate of Synthesis of o-Aminophenyl Glucuronide. The metal requirements for the glucuronidation of *o*-aminophenol are different from those for the other substrates as delineated above. In common with assays of UDP-glucuronyltransferase with *p*-nitrophenol, Mn^{2+} increases the activity at V_{max} of the enzyme with *o*-aminophenol as aglycone (Figure 4). In contrast to the results with *p*-nitrophenol, EDTA inhibits the rate of synthesis of *o*-aminophenyl glucuronide in microsomes prepared in 0.25 M sucrose (Table V). Also, unlike the results obtained with *o*-aminobenzoate and *p*-nitrophenol as aglycones, UDP-*N*-acetylglucosamine increases the rate of synthesis of *o*-aminophenyl glucuronide in the absence of metal ions (Table V). Thus, EDTA decreases the rate of synthesis of *o*-aminophenyl

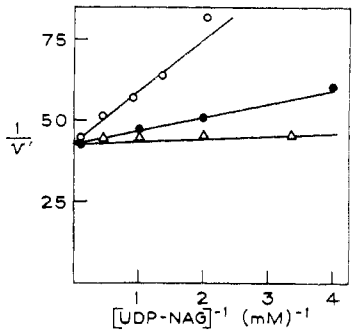

FIGURE 3: The rate of glucuronidation of *o*-aminobenzoate as a function of the concentration of UDP-*N*-acetylglucosamine (UDP-NAG) at variable fixed concentrations of Mn^{2+}. The rate of synthesis of *o*-aminobenzoyl glucuronide was determined as in Methods in microsomes from livers homogenized in sucrose–EDTA. The concentration of *o*-aminobenzoate was 0.2 mM, UDP-glucuronic acid, 1.0 mM, and of Mn^{2+}, 0.05 mM (○), 0.25 mM (●), or 0.5 mM (△). Each rate plotted is the rate (v') corrected for the fact that *o*-aminobenzoyl glucuronide is synthesized in the absence of UDP-*N*-acetylglucosamine. This was done by subtracting the rate in the absence of UDP-*N*-acetylglucosamine from the rates in the presence of the indicated concentrations of this compound. Units of activity are the same as in Figure 1.

glucuronide, but the ratio of activity in the presence to that in the absence of UDP-*N*-acetylglucosamine is independent of the concentration of EDTA, up to the levels as high as 10 mM.

The $K_{0.5}$ of UDP-*N*-acetylglucosamine is 0.023 mM for assays of UDP-glucuronyltransferase with *o*-aminophenol as aglycone. This is important for function not only because the binding of UDP-*N*-acetylglucosamine decreases the $K_{0.5}$ of UDP-glucuronic acid, but also because UDP-*N*-acetylglucosamine decreases the concentration of Mn^{2+} needed for half-maximal stimulation of the rate of synthesis of *o*-aminophenylglucuronic acid from 2.5 to 0.06 mM (Figure 5).

Discussion

Site of Action of Metal Ions. One of many difficulties encountered in studies of enzymes bound firmly to membranes is determining the site of action of effectors. Two considerations which apply to the data presented above are the possibilities that metals interact with substrate rather than with the enzyme or that metals alter the activities of UDP-glucuronyltransferase as a result of nonspecific effects on the membrane. Since there is no absolute metal requirement, there is no evidence that metal ions chelated to UDP-glucuronic acid are the true substrates in glucuronyltransferase reactions. Further, the differential effects of metals on the rates of synthesis of various glucuronides are inconsistent with nonspecific interactions between metals and portions of the membrane other than UDP-glucuronyltransferases. Moreover, studies with the nitroxide-labeled spin probes, 4 and 12 nitroxide stearate, indicate that concentrations of divalent ions which are modifiers of the activity of UDP-glucuronyltransferases do not perturb the lipid structure of the membrane.[1] For these reasons, we consider it reasonable to conclude that metal ions modify UDP-glucuronyltransferase as a result of direct binding to the enzyme.

[1] D. A. Vessey, S. Eletr, and D. Zakim, unpublished observations.

TABLE V: Effect of EDTA on the Rate of Synthesis of *o*-Aminophenylglucuronic Acid.[a]

Concn of EDTA (mM)	Act. of Unstimulated Enzyme	Act. of UDP-*N*-acetyl-glucosamine Stimulated Enzyme	Ratio of Act. in Presence and Absence of UDP-*N*-acetyl-glucosamine
0	1.5	2.73	1.82
0.5	0.88	2.10	2.40
2.5	0.78	1.50	1.92
5	0.56	1.12	2.00
10	0.49	0.98	2.00

[a] Initial rates of activity of UDP-glucuronyltransferase in microsomes from liver homogenized in 0.25 M sucrose were determined as in Methods as a function of the indicated concentrations of EDTA. The concentrations of substrates in each assay were 0.2 mM for *o*-aminophenol and 1.0 mM for UDP-glucuronic acid. When added, the concentration of UDP-*N*-acetylglucosamine was 2.0 mM. Units of activity are nmol of *o*-aminophenylglucuronide synthesized per min per mg of protein.

Differentiation of Classes of UDP-glucuronyltransferase Catalyzed Reactions According to Their Regulatory Parameters. Comparisons of the effects of metal ions on the activity of UDP-glucuronyltransferase with *o*-aminobenzoate and *p*-nitrophenol as aglycones indicate that the glucuronidation of these compounds is not catalyzed by identical portions of a single protein. As we have shown previously, kinetic experiments also suggest that there are separate aglycone binding sites for *p*-nitrophenol, *o*-aminophenol, and *o*-aminobenzoate (Zakim *et al.*, 1973a). Thus, although the exact relation between the proteins catalyzing the synthesis of *o*-aminobenzoyl and *p*-nitrophenyl glucuronides is still uncertain, it is reasonable to refer to *o*-aminobenzoic-, *o*-aminophenol-, and *p*-nitrophenol-metabolizing forms of UDP-glucuronyltransferase.

The present data indicate not only that there are substrate-specific aglycone binding sites within the microsomes, but also that the regulatory properties of UDP-glucuronyltransferase catalyzed reactions differ according to the aglycone studied. There are at least three regulatory types. For the type I enzyme, metal ions increase activity at V_{max} and are required for activation by UDP-*N*-acetylglucosamine (*p*-nitrophenol-metabolizing form). For the type I_a enzyme, metal ions also increase activity at V_{max}, but there is no apparent metal requirement for the stimulation by UDP-*N*-acetylglucosamine (*o*-aminophenol-metabolizing form). For the type II enzyme, metal ions decrease the $K_{0.5}$ of the enzyme for UDP-glucuronic acid, and metals are required for stimulation by UDP-*N*-acetylglucosamine (*o*-aminobenzoate-metabolizing form). These results not only underline that not all UDP-glucuronyltransferase catalyzed reactions take place on identical parts of a single type of protein, but they also facilitate the experimental approach to determining the number of substrate-specific forms of these enzymes, and the basis for differences in specificity. Preliminary work along these lines indicates, for

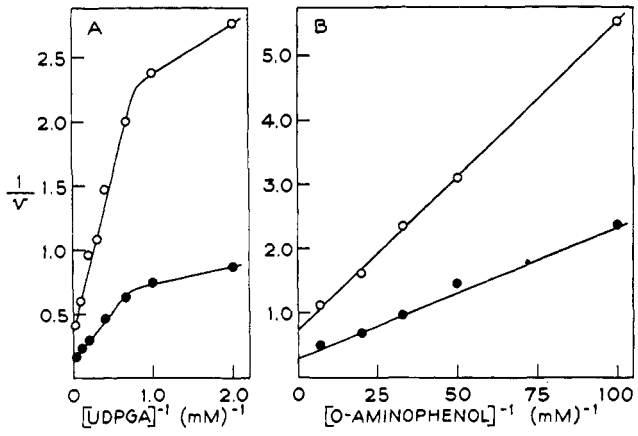

FIGURE 4: The effect of Mn^{2+} on the rate of synthesis of *o*-aminophenyl glucuronide. Initial rates of activity of UDP-glucuronyltransferase were measured with *o*-aminophenol as aglycone, as in Methods, as a function of the concentration of UDP-glucuronic acid (A) or *o*-aminophenol (B). Microsomes were from livers homogenized in sucrose–EDTA. In (A) the concentration of *o*-aminophenol was fixed at 0.2 mM; and in (B) the concentration of UDP-glucuronic acid was fixed at 2.5 mM. In both (A) and (B) assays were carried out in the absence (○) or presence (●) of 1.0 mM Mn^{2+}. Units of activity were nmol of *o*-aminophenyl glucuronide synthesized per min per mg of microsomal protein.

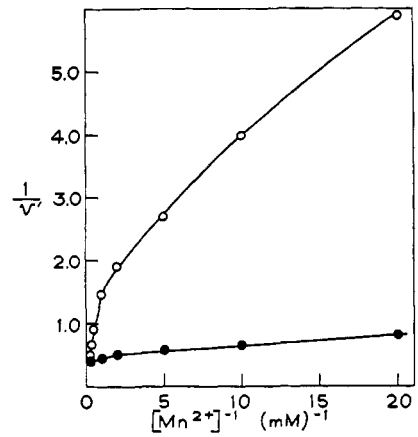

FIGURE 5: The rate of synthesis of *o*-aminophenylglucuronic acid as a function of the concentration of Mn^{2+}. The activity of UDP-glucuronyltransferase in microsomes from livers homogenized in sucrose–EDTA was determined as in Methods and Table V. Assays were carried out in 0.2 mM *o*-aminophenol, 1.0 mM UDP-glucuronic acid, and either 2.0 mM UDP-*N*-acetylglucosamine (●) or no UDP-*N*-acetylglucosamine (○); v' is the rate corrected, as in Figure 3, for the fact that *o*-aminophenol is glucuronidated in the absence of Mn^{2+}. Units are as in Figure 4.

example, that all derivatives of phenol tested are glucuronidated by enzymes of the type I or type I_a class.[2]

The need for several substrate-specific forms of UDP-glucuronyltransferase is apparent from examination of the variability in structure and reactivity of the compounds which are detoxified by glucuronidation. It is less clear why these enzymes have differing regulatory properties. Comparisons of the properties of the three regulatory types suggest, however, that the differences represent modifications of efficiency for detoxification, reflecting perhaps evolutionary changes. For example, as a result of interactions between the binding sites for divalent anions and UDP-*N*-acetylglucosamine, micromolar quantities of divalent ions and UDP-*N*-acetylglucosamine can effect a greater than 20-fold rate enhancement for glucuronidation of *o*-aminobenzoate at 0.2 mM UDP-glucuronic acid (type II enzyme). In the absence of interactions between the binding of metals and UDP-*N*-acetylglucosamine, in the *p*-nitrophenol-metabolizing form of the enzyme, millimolar quantities of metals and UDP-*N*-acetylglucosamine are needed in order to achieve rate enhancements of a similar extent (type I enzyme). For the *o*-aminophenol-metabolizing form of the enzyme (type I_a), no metal ions are required for stimulation by UDP-*N*-acetylglucosamine and the affinity of the enzyme for this ligand is quite high as compared to the other types of UDP-glucuronyltransferase. Also, in the type I_a enzyme, UDP-*N*-acetylglucosamine enhances the affinity of the enzyme for the binding of the metal ion, thereby potentiating the effect of a given concentration of metal on activity at V_{max}.

Physiological Significance of Regulation of UDP-glucuronyltransferase by Metal Ions. Modulation of the $K_{0.5}$ of UDP-glucuronic acid for UDP-glucuronyltransferase by other ligands could be critical for efficient detoxification *via* glucuronidation reactions because of the relatively low *in vivo* concentration of this substrate. Since the primary purpose of UDP-glucuronyltransferase catalyzed reactions is to detoxify endogenous and exogenous compounds, effectors of the reaction should ensure rapid rates of glucuronidation in order to prevent the accumulation of potentially toxic substrates. It cannot be stated with certainty that the concentrations of UDP-*N*-acetylglucosamine and UDP-glucuronic acid do not change significantly on a moment to moment basis; but it is unlikely that the concentrations of divalent cations change appreciably within the liver cell. Regulation by metal ions, especially in combination with UDP-*N*-acetylglucosamine is, therefore, suited to maintaining high rates of glucuronidation despite relatively small and perhaps fluctuating concentrations of UDP-glucuronic acid and UDP-*N*-acetylglucosamine. The effectors for UDP-glucuronyltransferase catalyzed reactions seem to poise these enzymes to detoxify aglycones rapidly, as they become available.

[2] D. Zakim, J. Goldenberg, and D. A. Vessey, unpublished observations.

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Synthesis and Characterization of Photoaffinity Labels for Adenosine 3′:5′-Cyclic Monophosphate and Adenosine 5′-Monophosphate†

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ABSTRACT: The synthesis and characterization of three ethyl-2-diazomalonyl derivatives of adenosine 3′:5′-cyclic monophosphate ($O^{2'}$-, N^6-, and N^6,$O^{2'}$-di) and of $O^{2'(3')}$-(ethyl-2-diazomalonyl)adenosine 5′-monophosphate are described. These derivatives have potential utility as photoaffinity labels. The N^6 derivatives are subject to pH-dependent Dimroth rearrangements, which imposes some constraints on their utility. From measurements of the rates of rearrangement it is concluded that at room temperature these derivatives can be used at pH 7, while at 5° the useful pH range is extended to pH 8. The rates of rearrangement of ethyl-2-diazomalonyl derivatives of aniline and benzylamine are also measured and are found to be very slow. It is concluded that the Dimroth rearrangement will not pose a general problem for the use of ethyl-2-diazomalonyl derivatives of amines as photoaffinity labels.

Adenosine 3′:5′-cyclic monophosphate (cAMP)[1] appears to play a role in a wide variety of biological processes (Robison *et al.*, 1971) and much current research is devoted to the isolation and characterization of the primary cAMP receptors in cells (Gill and Garren, 1971; Lemaire *et al.*, 1971; Maeno *et al.*, 1971; Walsh *et al.*, 1971; Anderson *et al.*, 1972; Rubin *et al.*, 1972). One approach to this problem is to covalently label the cAMP receptor in the intact cell. The advantages of using photoaffinity reagents for such an approach have been discussed previously (Kiefer *et al.*, 1970; Brunswick and Cooperman, 1971; Knowles, 1972). Two principal points are (1) that the reagents only become reactive on photolysis, so that they may be allowed to become fully equilibrated with the target protein before being activated for covalent bond formation; and (2) that the photolytically generated species are reactive enough to insert into any nearby bond, so that nucleophiles are not required to be present at the site for labeling to be achieved. In this paper we report the synthesis and characterization of three ethyl-2-diazomalonyl derivatives of cAMP of potential use as photoaffinity reagents. Two of these derivatives are substituted on the N^6 position and undergo pH-dependent Dimroth rearrangements. The apparent pK_a and rates of equilibration for this rearrangement have been studied, in order to assess what constraints the rearrangement imposes on the use of the N^6 derivatives as photoaffinity reagents. *N*-(Ethyl-2-diazomalonyl) derivatives of aniline and benzylamine have also been studied to investigate the general importance of the phenomenon for α-diazoamides.

Because of point 2 above, photoaffinity reagents should also be of great utility in characterizing binding sites, such as allosteric sites, even on purified proteins. In this paper we also describe the synthesis and characterization of $O^{2'(3')}$-(ethyl-2-diazomalonyl)adenosine 5′-monophosphate, a potential photoaffinity reagent for adenosine 5′-monophosphate, which is an allosteric effector of a large number of enzymes (Monod *et al.*, 1965).

Studies on the applications of these reagents have been or will be presented elsewhere (Brunswick and Cooperman, 1971; Cooperman and Brunswick, 1973; Guthrow *et al.*, 1973). A preliminary account of part of this work has been published previously (Brunswick and Cooperman, 1971).

Materials and Methods

Adenosine 3′:5′-cyclic monophosphate was obtained from Sigma. [^{3}H]Adenosine 3′:5′-cyclic monophosphate was obtained from New England Nuclear. Beef heart 3′:5′-cyclic nucleotide phosphodiesterase was obtained from Sigma. Ethyl-2-diazomalonyl chloride was prepared as described previously (Brunswick and Cooperman, 1971). *N*-(Ethyl-2-diazomalonyl)aniline was prepared as described by Leffler and Liu (1956). *N*-(Ethyl-2-diazomalonyl)benzylamine was prepared as described by Hoover and Day (1956).

Ascending paper chromatography was performed on either Whatman No. 40 or 3MM (preparative) paper, with an overnight development, using ethanol–0.5 M ammonium acetate (pH 7.0) (5:2, v/v) as solvent. Ascending thin layer chromatography (tlc) was carried out on Macherey-Nagel (Brinckmann)

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[1] Abbreviation used is: cAMP, adenosine 3′:5′-cyclic monophosphate.