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Observations of the effect of diethylnitrosamine on glucuronide formation

Glucuronides are formed enzymatically, *in vivo* and *in vitro*, by the transfer of glucuronic acid from UDP-glucuronic acid to a wide range of acceptors in the presence of UDP-glucuronyltransferase (UDP-glucuronate glucuronyltransferase, EC 2.4.1.17), a microsomal enzyme mainly found in liver. In studies of this enzyme *in vitro* the acceptor *o*-aminophenol is often used, since *o*-aminophenol glucuronide formed may be measured in the presence of excess *o*-aminophenol by the sensitive method of LEVVY AND STOREY[1]. GREENWOOD AND STEVENSON[2] demonstrated that the formation of *o*-aminophenol glucuronide by rat liver preparations *in vitro* was increased by the administration of the hepatocarcinogen, diethylnitrosamine, *in vivo* and that the addition *in vitro* of this agent to rat liver homogenate and microsomal suspensions also increased glucuronide formation. Further studies[3] revealed that the stimulatory effect of diethylnitrosamine is limited to the liver of rats and the glucuronide acceptors *o*-aminophenol and paracetamol. The effect is most striking in liver preparations from the GUNN[4] rat which has a persistent unconjugated hyperbilirubinaemia and low hepatic UDP-glucuronyltransferase activity to a wide range of acceptors[5]. The presence of diethylnitrosamine in assays from this strain of rat increases apparent *o*-aminophenol glucuronide formation to the same enhanced level found in similarly treated preparations from normal rats. In this study we report that *o*-aminophenol glucuronide formation is strikingly increased *in vitro* by diethylnitrosamine in assays with crude and partially purified hepatic UDP-glucuronyltransferase preparations[6] from hypophysectomized and thyroidectomized rats as well as Gunn rats. The effect of diethylnitrosamine is further investigated and evidence presented which suggests that its action is on the enzyme preparation rather than on the substrate.

Diethylnitrosamine, UDP-glucuronate as ammonium salt, both stated to be 98–100% pure, were obtained from Sigma Chemical Co. St. Louis, Mo., U.S.A.. *o*-Aminophenol was resublimed from commercially available *o*-aminophenol. *o*-Aminophenol glucuronide was a gift from Professor R. T. Williams. All other chemicals were obtained from commercial sources in the highest purity available and not further purified. Animals used in this study were as previously described[6]. The cat and goat specimens were obtained from healthy animals of unknown pedigree. These latter animals were killed with intravenous sodium pentobarbital. All others were killed by cervical dislocation. Livers were rapidly removed and a 25% homogenate in 0.14 M

KCl, 0.01 M Tris (pH 7.4) prepared using a mechanically powered Teflon-glass homogenizer. This and all subsequent procedures were carried out at 0–4°. More purified enzyme preparations, *viz.* particulate suspension, dialyzed particulate fraction and Sephadex G-200 eluate were prepared as in earlier studies[6]. UDP-glucuronyltransferase was determined using previously described techniques[6] with *o*-aminophenol, bilirubin, anthranilic acid *p*-nitrophenol and methylumbelliferone as glucuronide acceptors.

Protein concentration was determined by the method of LOWRY *et al.*[7] and serum bilirubin by the MALLOY AND EVELYN[8] technique.

8 mM diethylnitrosamine did not change the pH of the standard *o*-aminophenol assay or of the incubation mixture prior to diazotization, nor did this concentration enhance color production when added to authentic *o*-aminophenol glucuronide. It had no stabilizing effect on *o*-aminophenol glucuronide incubated with particulate fractions from livers of Wistar or Gunn rats.

No interaction between *o*-aminophenol and diethylnitrosamine was observed when solutions were studied using a Cary spectrophotometric recording absorptiometer. *o*-Aminophenol absorption (maximal at 263 nm) decreased following incubation at 37° for 20 min. This decrease was not influenced by the presence of diethylnitrosamine (maximal absorption at 228 nm) when the compounds were incubated together in a molar ratio similar to that in a standard assay containing 8 mM diethylnitrosamine. No new absorption peaks appeared during incubation.

Preincubation of Gunn rat liver particulate fraction with 8 mM diethylnitrosamine at 0° for 1 h and subsequent incubation with *o*-aminophenol and UDP-glucuronate at a final diethylnitrosamine concentration of 1.4 mM resulted in greater *o*-aminophenol glucuronide formation than an assay similarly preincubated but containing only 1.4 mM diethylnitrosamine throughout. Incubation of Gunn rat liver particulate fraction with 8 mM diethylnitrosamine at 4° for 1 h, centrifugation at 100 000 × *g* for 45 min at 4°, resuspension in 0.14 M KCl, 0.01 M Tris–HCl (pH 9.0), with repeat centrifugation and resuspension resulted in the absence of enhanced *o*-aminophenol glucuronide formation, but this was recovered when 8 mM diethylnitrosamine was added to the assay.

Although Gunn rat liver particulate fraction stored at 4° retained the ability to conjugate *o*-aminophenol in the presence of 8 mM diethylnitrosamine for at least 4 days whether diethylnitrosamine was present throughout storage or only added at the time of assay, dialysis of a particulate suspension containing 8 mM diethylnitrosamine against 100 vol. of 0.1 M EDTA and 1.0 mM mercaptoethanol (pH 9.0) for 18 h at 4° caused irreversible loss of conjugating ability. This did not occur when particulate fraction without diethylnitrosamine was dialyzed in the same conditions. The same experiment with a Wistar rat liver preparation revealed that the enhancement of *o*-aminophenol conjugation by diethylnitrosamine disappeared similarly after dialysis.

Assays *in vitro* using liver particulate suspensions from guinea pig, goat and cat showed no increase in *o*-aminophenol conjugation when diethylnitrosamine was added to the assay. Similarly, there was no increase in *o*-aminophenol glucuronide formation when the enzyme source was kidney from normal Wistar rats, Gunn rats or hypophysectomized Sprague–Dawley rats. When bilirubin, *p*-aminobenzoic acid, *p*-nitrophenol or methylumbelliferone were substrates the addition of diethylnitrosamine did not increase conjugation in studies using Wistar or Gunn rat liver particulate suspensions as enzyme source.

TABLE I

EFFECT OF DIETHYLNITROSAMINE ON SERUM BILIRUBIN CONCENTRATION IN ADULT MALE GUNN RATS (170–200 g)

Drug was given intraperitoneally (30 mg/kg) in a once daily injection. Control animals received N saline. Data are means ± 1 S.E. Figures in parentheses indicate number of animals studied.

Day of treatment	*Serum bilirubin (mg/100 ml)*	
	Saline treated (4)	*Drug treated (4)*
−4	3.1 ± 0.4	4.1 ± 0.3
−1	4.4 ± 0.1	4.4 ± 0.1
+4	4.2 ± 0.3	4.8 ± 0.1
+7	6.5 ± 1.3	6.5 ± 0.5
+11	6.3 ± 1.1	9.6 ± 2.1

TABLE II

ACTIVATION OF LIVER UDP-GLUCURONYLTRANSFERASE *in vitro* BY DIETHYLNITROSAMINE (8 mM) WITH *o*-AMINOPHENOL AS GLUCURONIDE ACCEPTOR

Figures in parentheses indicate number of animals studied.

Animal	*Enzyme preparation*	*o-Aminophenol glucuronide formed (nmoles/mg protein per 20 min)*		*Activation*
		0 mM diethylnitrosamine	*8.0 mM diethylnitrosamine*	
Intact Sprague–Dawley rat (4)	25% homogenate	1.5	3.7	2.4
	Total particulate suspension	9.0	20.0	2.2
	Dialyzed particulate fraction	23	54	2.3
	Sephadex G-200 eluate	27	58	2.1
Hypophysectomized Sprague–Dawley rat (6)	25% homogenate	0.6	3.0	5.0
	Total particulate suspension	0.6	24.0	40.0
	Dialyzed particulate fraction	1.0	22	22
	Sephadex G-200 eluate	3.0	38	12.6
Thyroidectomized Sprague–Dawley rat (3)	25% homogenate	0.5	3.0	6.0
	Total particulate suspension	0.8	5.2	6.0
	Dialyzed particulate fraction	3.5	27	8.0
	Sephadex G-200	4.0	39	9.7
Thyroxine-treated hypophysectomized Sprague–Dawley rat (2)	25% homogenate	1.8	6.1	3.4
	Total particulate	7.7	25	3.0
	Dialyzed particulate fraction	—	—	—
	Sephadex G-200	—	—	—
Gunn rat (6)	25% homogenate	0.2	2.5	12
	Total particulate suspension	0.5	5.4	10
	Dialyzed particulate fraction	1.0	26	26
	Sephadex G-200	3.0	83	28

The effect of diethylnitrosamine on the serum bilirubin concentration in the Gunn rat *in vivo* is shown in Table I. Its failure to lower serum bilirubin levels is in keeping with its failure to enhance bilirubin conjugation *in vitro*. The rise in serum bilirubin was associated with a fall in liver weight from a mean of 8.4 g in the control group to 4.0 G in the treated group.

The studies reported in Table II show that the limited ability of liver preparations from hypophysectomized and thyroidectomized rats to conjugate *o*-aminophenol with glucuronic acid *in vitro* is increased to an extent similar to that observed in Gunn rats, and illustrate the role of thyroid hormone in this phenomenon. The administration of thyroxine *in vivo* to hypophysectomized rats in a daily dose of 10 μg subcutaneously for 10 days increased the conjugation of *o*-aminophenol *in vitro* towards normal levels and decreased the activation caused by diethylnitrosamine.

These results indicate that the enhanced *o*-aminophenol glucuronide formation *in vitro* in systems containing diethylnitrosamine is due to its action on some constituent of the particulate suspension which is under hormonal influence. Limitation of this effect of diethylnitrosamine to two substrates in only one tissue in a single species is consistent with its action being limited to a specific UDP-glucuronyltransferase peculiar to the rat liver. It is equally possible that the effect may be on nonenzymatic components of the impure enzyme preparations studied[6]. Further investigation of the effects of dialysis in alkaline EDTA in normal, hypophysectomized or thyroidectomized rats, with and without thyroxine treatment, may resolve this problem and provide further information on the possible multiplicity of UDP-glucuronyltransferase.

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