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GLUCURONIDATION OF MONO(2-ETHYLHEXYL)PHTHALATE

SOME ENZYME CHARACTERISTICS AND INHIBITION BY BILIRUBIN

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Abstract—A method for assaying mono(2-ethylhexyl)phthalate (MEHP) uridine diphosphate (UDP) glucuronyl transferase activity in microsomal preparations from guinea pig liver is described. The quantitation of the MEHP-glucuronide was performed by HPLC after direct injection of a sample of deproteinized incubation mixture. Solubilization of microsomal UDP-glucuronyltransferase activity was achieved by use of Lubrol, and optimal conditions for glucuronidation of MEHP were established. To investigate whether there is competition between MEHP and bilirubin for glucuronidation, inhibition experiments were performed with solubilized enzyme preparations. In these incubations addition of bilirubin decreased the formation of MEHP-glucuronide. No change in the maximal conversion rate (V_{max}) was observed, indicating the occurrence of competitive inhibition. This observation may have implications in clinical situations where patients with hyperbilirubinemia are exposed to MEHP, e.g. in exchange transfusions in newborn infants.

Exposure to the plasticizer di(2-ethylhexyl)phthalate (DEHP) may occur during a number of situations in medical care where blood products or lipophilic solutions have been in contact with polyvinyl chloride material [1]. Examples of situations where the exposure to DEHP may be of possible significance are exchange transfusions of newborn infants with hyperbilirubinemia, and haemodialysis of patients with renal failure [2, 3].

It is well established that the toxicity of DEHP is not exerted by DEHP itself, but mainly by its mono-deesterified metabolite, mono(2-ethylhexyl)phthalate (MEHP) [4]. This compound has been detected in considerable quantities in the blood of patients exposed to DEHP [2, 3]. In fact, it can be formed *in vitro* during the storage of blood or blood products by the action of non-specific plasma esterases [5].

In experimental animals and in man, elimination of the metabolite MEHP may occur by oxidation or glucuronidation [6]. Since bilirubin is metabolized by glucuronidation, the presence of high circulating blood levels of MEHP in hyperbilirubinemic newborn infants subjected to exchange transfusions [2] raises the question of whether there is competition between MEHP and bilirubin for the same glucuronyltransferase system(s). Such competition may lead to prolonged exposure to MEHP and/or bilirubin. In this paper we describe a method for assaying the formation of MEHP-glucuronide *in vitro*. Using this method, we have studied some characteristics of the glucuronyltransferase system active on MEHP, including the possible competition between MEHP and bilirubin with regard to glucuronidation. On the basis of observations that guinea pigs eliminate MEHP mainly by glucuronidation [6], solubilized preparations from guinea pig liver microsomes were chosen for the experiments.

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MATERIALS AND METHODS

[^{14}C]Mono(2-ethylhexyl)phthalate ([^{14}C]MEHP) was synthesized from carbonyl-[^{14}C]phthalic anhydride (500 μCi, 58 mCi/nmol, Amersham International plc, Amersham, Bucks, U.K.) and 2-ethyl-1-hexanol (0.02 nmol, Berol Kemi, Stenungsund, Sweden) by heating the two compounds in 100 μL of toluene at 110° for 6 hr. The reaction mixture was separated by preparative thin layer chromatography (TLC) (SiO_2, 60, F_{254}, 0.25 mm with concentration zone, Merck, Darmstadt, F.R.G.) with dichloromethane/ethyl acetate, 1:1 (v/v) as the mobile phase. Radiochemically pure [^{14}C]MEHP (414 μCi) was obtained and compared with unlabelled MEHP by TLC. Unlabelled MEHP was synthesized according to a similar procedure [7].

[^{14}C]MEHP-glucuronide was obtained from urine of guinea pigs in which [^{14}C]MEHP had been administered. This species was chosen since it is known to excrete a large fraction of the administered dose as glucuronide [6]. [^{14}C]MEHP was diluted with unlabelled MEHP to give a specific activity of 3.8 μCi/nmol, and two guinea pigs were each given 1.4 nmol/kg body weight of the mixture. Urine was collected on ice for up to 24 hr and the pH of the urine was adjusted to approximately 5. The MEHP-derived metabolites were extracted with octadecylsilane-bonded silica. The extract was

purified by ion-exchange chromatography followed by reversed-phase HPLC [8].

The purity of the [^{14}C]MEHP-glucuronide was checked by HPLC, using a Nova-Pak C_{18} (8 mm × 10 cm) Radial Pak cartridge in a Z-module (Waters Milford, MA, U.S.A.) with a mobile phase of 45% aqueous acetonitrile, containing 0.1% trifluoroacetic acid, and with a UV detector set at 254 nm. TLC was performed in 1-butanol/acetic acid/water, 10:1:2 (v/v/v) and spots were viewed under UV light (254 nm) and visualized by spraying with 50% sulphuric acid or spraying with chromic acid followed by heating. The [^{14}C]MEHP-glucuronide was properly characterized by means of fast atom bombardment mass spectrometry (exact mass determination) and was found to yield MEHP when hydrolysed with β-glucuronidase/sulphatase [8]. The purity was better than 98% as determined by HPLC, and only one spot was observed with TLC (R_f = 0.35). Separate experiments showed that MEHP-glucuronide is stable between pH 6.4–7.8.

All solvents used were of at least analytical grade. Lubrol, uridine 5'-diphospho(UDP)glucuronic acid, bilirubin and D-saccharic acid 1,4-lactone (saccharolactone) were obtained from the Sigma Chemical Company (St Louis, MO, U.S.A.).

Animals. Male guinea pigs (Dunkin Hartley) were obtained from Sahlins Försöksdjursfarm (Malmö, Sweden). They weighed 250–300 g and were killed by cervical dislocation and decapitation.

Preparation of liver microsomes and solubilized fraction. Liver tissue was excised and pooled samples were placed in ice-cold 0.25 M sucrose. Ten per cent homogenates (w/v) were prepared with a Potter-Elvehjem homogenizer equipped with a loosely fitting pestle. The microsomal fraction was obtained from the homogenate by centrifugations at 800, 20,000 and 100,000 *g*. A small volume of 0.25 M sucrose was laid over the microsomal pellet, which was then kept frozen at −20° for up to 2 months before use. Solubilized microsomes were prepared by thawing the microsomal pellet and removing the sucrose overlay. The pellet was washed several times in 0.1 M imidazole–HCl buffer (pH 6.8) and a suspension (protein concentration between 1.5 and 2 mg/mL) was prepared by gentle homogenization in the same buffer. After the addition of 0.1% (v/v) Lubrol, the mixture was stirred on ice for 30 min, after which it was centrifuged at 100,000 *g* for 1 hr. The supernatant was mixed with XAD-2 (8 g/100 mL enzyme solution), which was then removed by filtration. The protein concentration in the solubilized preparations was between 1 and 2 mg/mL as determined by the method of Bradford [9].

Incubations. In standard incubations with the microsomal fraction, 0.25 mg of protein, with or without the addition of 0.1% (v/v) Lubrol, was incubated with 0.27 mM of MEHP at 37° for 20 min in 0.1 M imidazole–HCl buffer (pH = 6.8). Each incubation contained 5.0 mM each of UDP-glucuronic acid and saccharolactone. The reactions were terminated by heating the samples in a boiling water bath for 45 sec.

The effect of time and of the amount of protein, UDP-glucuronic acid and substrate on the formation of MEHP-glucuronide was investigated with both the microsomal fraction and the solubilized fraction. The standard incubations with the solubilized fraction were performed similarly to those with the microsomal fractions with regard to total volume, incubation time, and amounts of substrate and protein. These incubations were supplemented with 4.3 μmol of UDP-glucuronic acid.

In experiments with the solubilized fraction, the inhibition of MEHP-glucuronidation by bilirubin was investigated according to the Lineweaver–Burk method. In these experiments bilirubin in 0.1 mL of ethylene glycol containing 1% (v/v) of ethanolamine was added to the incubation mixtures. To minimize the effect of light on bilirubin, the incubations were carried out in the dark.

Assay procedure. After termination of the incubations, the samples were put on ice, sonicated, and centrifuged for 5 min at 1000 *g*. The content of MEHP-glucuronide in the supernatant was determined in a Waters liquid chromatographic system consisting of a model 501 pump, model 481 UV detector and a Nova-Pak C_{18} steel column (Waters). MEHP-glucuronide and MEHP were eluted with a mixture of acetonitrile–water–acetic acid, 49:50:1 (v/v/v) at a flow rate of 1 mL/min. The eluted MEHP-glucuronide, which had a retention time of 4 min, was quantitated by UV absorption at 280 nm. Calibration curves were made by the addition of known amounts of MEHP-glucuronide to the incubation mixtures followed by treatment as described above. The calibration curves were linear over the concentrations studied. All samples were analysed within 24 hr after incubation. It was ascertained that there was no degradation or isomerization of MEHP-glucuronide during this time period.

RESULTS

Optimal conditions were established for the glucuronidation of MEHP in the microsomal fraction with and without the addition of 0.1% (v/v) Lubrol (not shown). The reaction was linear with time for at least 40 min and with an amount of microsomal protein of up to 0.23 mg. Substrate saturation was obtained at a concentration of 0.27 mM MEHP and the maximal conversion rate was observed at a UDP-glucuronic acid concentration of approximately 5 mM. The optimal pH of the reaction was found to be 6.7–6.8.

Direct addition of 0.1% (v/v) Lubrol to the microsomal fraction increased the conversion rate more than two-fold, from 5 to 12 nmol/min/mg protein. The presence of enzyme activity in the supernatant obtained after centrifugation at 100,000 *g* proved that enzyme solubilization had occurred. When the effects of different concentrations of Lubrol on solubilization and enzyme activity was investigated, higher activity was obtained with 0.1% (v/v) Lubrol as compared to either 0.01, 0.05 or 0.5% (v/v) of the detergent.

Under optimal conditions glucuronidation of MEHP was somewhat lower in a 100,000 *g* supernatant fluid than in a microsomal preparation, 3.8 as against 5 nmol/min/mg of protein. Figure 1 shows the optimal conditions for the glucuronidation of

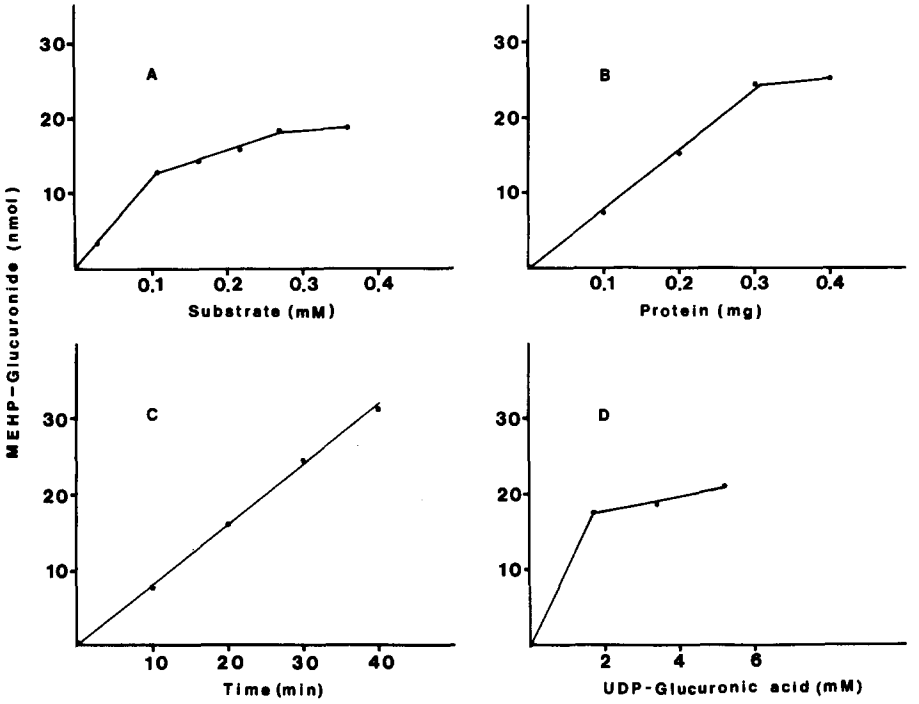

Fig. 1. (A–D) The effect of time and of the amounts of protein, substrate and UDP-glucuronic acid on the formation of MEHP-glucuronide in 100,000 *g* supernatant fluid from solubilized microsomal preparations. The conditions of the incubations were as described in Materials and Methods.

MEHP in the solubilized preparation. Substrate saturation was achieved at a concentration of 0.2 mM MEHP. The reaction was linear with a protein amount of up to 0.3 mg and with an incubation time of at least 40 min. Addition of UDP glucuronic acid, in a concentration of 2 mM, saturated the system.

Figure 2 shows a Lineweaver–Burk plot illustrating the inhibitory effect of various concentrations of bilirubin on the glucuronidation of MEHP (K_m = 0.085 M and V_{max} = 3.8 nmol/min/mg protein). Since the maximal conversion rate (V_{max}) was not altered by the presence of bilirubin, the occurrence of competitive inhibition is indicated.

DISCUSSION

Glucuronidation is a major mechanism for elimination of the potentially toxic compound MEHP, the primary metabolite of the plasticizer DEHP [6]. Knowledge of the efficiency and characteristics of this reaction is important in the understanding of the disposition of MEHP both in laboratory animals and in patients. The present work shows that solubilized preparations from guinea pig liver microsomes can be used for such investigations.

In similarity to other mammalian drug- and xenobiotic metabolizing enzyme systems, liver UDP-glucuronyltransferase activity involves several enzymes, partly with overlapping substrate specificities [10]. In the absence of results from studies with extensively purified enzyme preparations, it is not possible to tell whether only one or more

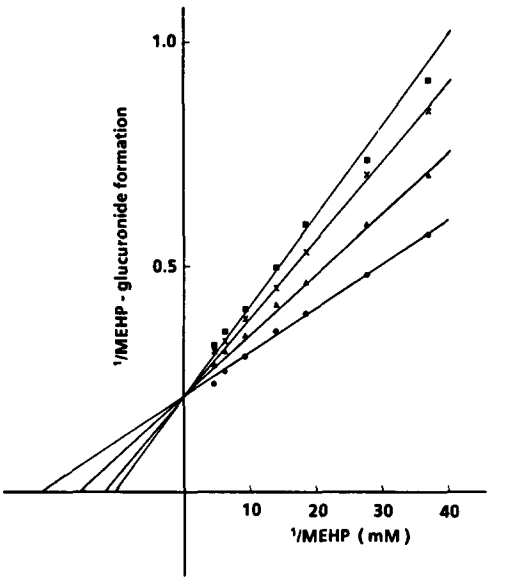

Fig. 2. A Lineweaver–Burk plot demonstrating the inhibitory effect of bilirubin (added in ethylene glycol containing 0.1% of ethanolamine) on MEHP-glucuronyltransferase activity (nmol/min/mg protein) in 100,000 *g* supernatant fluid from solubilized microsomal preparations. The bilirubin concentrations were 0 (●—●), 0.15 (▲—▲) and 0.25 (×—×) and 0.40 (■—■) mM. The conditions of the incubation were as described in Materials and Methods.

glucuronyltransferases are active in MEHP conjugation.

The simultaneous occurrence in the body of MEHP and other compounds utilizing the same enzyme system(s) for glucuronidation may lead to a decreased clearance of MEHP. We have previously identified hyperbilirubinemic newborn infants, undergoing exchange transfusions, to be in a situation where high levels of bilirubin and MEHP occurred simultaneously [2]. The present results indicating the occurrence of competitive inhibition between MEHP and bilirubin for glucuronidation, could thus be one explanation for the unusually high levels of MEHP found in these newborns. In addition, an inhibitory effect of MEHP on bilirubin glucuronidation could lead to a decreased elimination of bilirubin, thus partly counteracting the effect of the exchange transfusion.

Another type of interaction may take place when bilirubin and MEHP are present simultaneously at high concentrations. Both compounds are highly bound to serum albumin [11, 12], and the question arises as to whether MEHP is able to displace bilirubin from its binding site on albumin or vice versa. If so, this may be yet another reason for reducing or completely eliminating the inadvertent exposure to MEHP that occurs during exchange transfusions of newborn infants.

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