Biochemical Pharmacology, Vol. 27, pp. 2583–2588.
Pergamon Press Ltd. 1978. Printed in Great Britain.

INTERACTION OF ETHANOL OXIDATION WITH GLUCURONIDATION IN ISOLATED HEPATOCYTES

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(*Received* 18 *January* 1978; *accepted* 13 *March* 1978)

Abstract—1. Glucuronidation of harmol, 2-naphthol, 4-methylumbelliferone and phenolphthalein in isolated hepatocytes was inhibited up to 50 per cent in the presence of low concentrations of ethanol (10 mM). Sulphate conjugation was unaffected. The inhibitory effect of ethanol was reversed by 4-methylpyrazole, an inhibitor of alcohol dehydrogenase dependent ethanol oxidation. 2. The oxidation of harmine to harmol was not affected by 10 mM ethanol, but in hepatocytes isolated from phenobarbital-treated rats glucuronidation of the formed harmol was inhibited about 30 per cent in the presence of this amount of ethanol. 3. Ethanol increased the intracellular NADH/NAD^+ ratio as did lactate and sorbitol. The latter two substances were also inhibitory to glucuronidation having no effect on the sulphate conjugation. 4. The synthesis of UDPglucuronic acid was inhibited by ethanol both in the presence and absence of a substrate undergoing glucuronidation. It is suggested that the inhibitory effect of ethanol on glucuronidation is due to a decreased UDPglucuronic acid synthesis caused by the increased NADH/NAD^+ ratio resulting from the alcohol dehydrogenase dependent oxidation of ethanol.

Numerous studies have been concerned with the interaction between ethanol and the metabolism of drugs and other foreign compounds both *in vivo* [1–3] and *in vitro* [4–9]. The observed effects have most often been inhibitory and ascribed to a direct inhibitory action of ethanol on the microsomal mixed function oxidase pathway. The nature of this inhibitory action is not yet fully established but is generally believed to be due to interference of ethanol with the binding of the drug substrate to cytochrome P-450 either by competitive binding of ethanol [10] or displacement of bound drug substrate by ethanol [11].

Only a few studies have been concerned with the effect of ethanol on the conjugation reactions. Induction of UDPglucuronosyltransferase by ethanol has been suggested [12]. Inhibition of *p*-nitrophenol glucuronidation in microsomes by different alcohols [13] as well as a stimulatory effect by rather high ethanol concentrations on glucuronidation of various substrates, also in microsomes, have been observed [6]. A decreased rate of glucuronidation of disulfiram *in vivo* after administration of ethanol has also been reported [14].

We recently reported an inhibitory effect of ethanol on the conjugation of *p*-nitrophenol in isolated hepatocytes [15]. This effect, which was seen at limited ethanol concentrations, was apparently not due to a direct interaction of ethanol on the conjugation but resulted from the alcohol dehydrogenase dependent ethanol oxidation. It was suggested that the increased NADH/NAD^+ ratio, resulting from this ethanol oxidation, might interfere with the synthesis of UDPglucuronic acid by inhibiting the UDPglucose dehydrogenase catalyzed reaction. The purpose of the present study was to further investigate the nature of this interaction between ethanol and the conjugation reactions at the cellular level.

MATERIALS AND METHODS

Male Sprague–Dawley rats (200–250 g) were used. Phenobarbital was administered by three daily intraperitoneal injections of 80 mg per kg body weight. Isolation of hepatocytes was performed by collagenase perfusion as previously described [16]. The yield of the preparations was 2–4 $\times$ 10^8 cells per liver when determined in a Buerker chamber. Trypan blue and NADH exclusions were 90–100 per cent immediately after cell isolation.

Incubation of hepatocytes. Incubations were performed using substrate concentrations of 200 μM in Krebs–Henseleit buffer, pH 7.4, with 3 $\times$ 10^6 cells/ml incubation medium. Bovine serum albumin was omitted from the incubation mixture due to protein binding of the used substrates. All experiments were performed within two hours after cell preparations. Round-bottom tubes (30 ml) covered with parafilm were used for the incubations at 37° for 20 min. The incubation medium was gassed with 95% O_2–5% CO_2 immediately before the addition of cells, to ensure adequate oxygenation. No difference in the rate of conjugation was observed when compared to incubations in open tubes.

Analytical methods. Conjugation of phenolphthalein and 4-methylumbelliferone were measured essentially according to Mulder [17] and performed in the following way. The reactions were terminated by addition of 0.5 ml 0.3 M glycine–0.2 M TCA buffer, pH 2.2 per ml of incubation mixture. For phenolphthalein, 0.9 ml 0.4 M glycine NaOH buffer, pH 10.4, was added to 0.4 ml of the suspension prior to sedimentation of the precipitate. After sedimentation the decrease in absorbance at 555 nm was measured in the clear supernatant. For 4-methylumbelliferone 0.9 ml 0.4 M glycine–NaOH buffer, pH 10.4, was added to 0.4 ml of the supernatant after sedimentation

of the precipitate and the decrease in absorbance at 365 nm was measured.

Conjugation of 2-naphthol was determined in the same way as 4-methylumbelliferone, except that 0.4 ml of supernatant was added to 1.6 ml 1.0 M Tris–HCl buffer, pH 9.0, and the decrease in fluorescence was measured in an Aminco–Bowman spectrofluorometer using an excitation wavelength of 330 nm and an emission wavelength of 460 nm [18]. Blanks were obtained from incubations containing no substrate.

Phenolphthalein, 4-methylumbelliferone and 2-naphthol were added in a small amount of acetone to the incubation medium. No difference in metabolic activity was observed if the acetone was evaporated from incubation tube prior to addition of the incubation mixture, indicating that acetone had no inhibitory effect.

Metabolism of harmine and the conjugation of harmol was measured according to Mulder *et al.* [19] with minor modifications, and was carried out in the following way. The incubations were stopped by addition of 0.5 ml 0.3 M glycine–0.2 M TCA buffer, pH 2.2 per ml sample. Protein was sedimented by centrifugation, and 25 μl of the clear supernatant was applied to TLC-plates (silica gel). Chromatographic procedure was performed at room temperature in the dark, using chloroform:methanol:isopropanol:ammonia (90:10:95:5) as solvent system. The separated products were visualized with u.v., eluted with 3.0 ml 0.1 M HCl and quantified by fluorescence, using an excitation wavelength of 320 nm and an emission wavelength of 420 nm.

Determination of nicotinamide nucleotide levels. Incubations with 3×10^6 cells/ml were performed for 10 min at 37° as described above. Samples (1.0 ml) were withdrawn for analysis of the reduced and oxidized nicotinamide nucleotides according to Klingenberg [20].

UDPglucuronic acid (UDPGlcUA) determination in isolated hepatocytes. The procedure which is modified after Bock *et al.* [18] was carried out in the following way. 25 ml Erlenmyer flasks were used in which the cell suspensions were equilibrated in Krebs–Henseleit buffer, pH 7.4, under 95% O_2–5% CO_2 at 37° for 2 min. Incubations were started by addition of 12 mM ethanol and/or 200 μM 4-methylumbelliferone (final concentrations), and the flasks were covered with parafilm prior to incubations. 3.0 ml aliquots of the incubation mixture were pipetted into 1.5 ml ice cold 0.3 M glycine–0.2 M TCA buffer, pH 2.2, and the samples were homogenized before centrifugation at 10,000 g for 10 min. The supernatants were transferred to centrifuge tubes and 6 volumes (27 ml) −20° acetone was added. Incubations were carried out for 1 hr at −20°, and the precipitated UDPGlcUA was sedimented by centrifugation. Excess of acetone was removed by evaporation under vacuum at 4°, and the precipitate was dissolved in 0.7 ml distilled water. The UDPGlcUA content was determined by the glucuronidation of harmol as described above, using 400 μM substrate concentration and a final incubation volume of 1.0 ml containing 0.1 M Tris–HCl buffer, pH 7.4, 5 mM $MgCl_2$, microsomes (1 mg/ml final concentration) and 0.5 ml of extracted UDPGlcUA fraction. Incubations which were started by addition of microsomes prepared according to Ernster *et al.* [21] and activated by 0.2% Triton X-100 were performed for 1 hr at 37°. Formation of glucuronides was determined according to Mulder *et al.* [19].

Chemicals. Harmol hydrochloride was obtained from Aldrich-Europe (Beerse, Belgium) and harmine hydrochloride from EGA-Chemie KG (Steinheim-Albuch, Germany). 4-Methylpyrazole was a gift from Astra Pharmaceuticals (Södertälje, Sweden). Collagenase was purchased from Boehringer-Mannheim GmbH (Mannheim, Germany), and all other chemicals used were of analytical grade and obtained from local commercial sources.

RESULTS

Ethanol inhibited glucuronidation of harmol in isolated hepatocytes (Fig. 1A). A maximal inhibition of more than 40 per cent was observed at an ethanol concentration of 10 mM. Addition of 4-methylpyrazole, an inhibitor of alcohol dehydrogenase [22], reversed the ethanol effect. The reversal was not complete at higher than 10 mM ethanol concentration

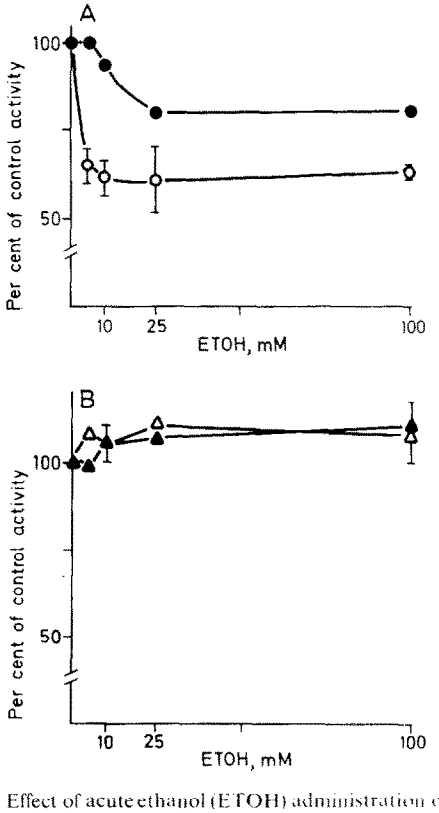

Fig. 1. Effect of acute ethanol (ETOH) administration on the conjugation of harmol in hepatocytes isolated from control rats. Incubations were performed as described in "Materials and Methods" in the presence (filled symbols) and absence (open symbols) of 1 mM 4-methylpyrazole. △, ○, ▲, ●. Glucuronides. A. △—△, ▲—▲. Sulphate conjugates. B. Values represent means ± S.E.M. of 2–3 different hepatocyte preparations. One hundred per cent activity represents for glucuronidation 24.6 nmol/10^6 cells/20 min and for sulphate conjugation 4.0 nmol/10^6 cells/20 min. Harmol concentration was 200 μM (67 nmol/10^6 cells).

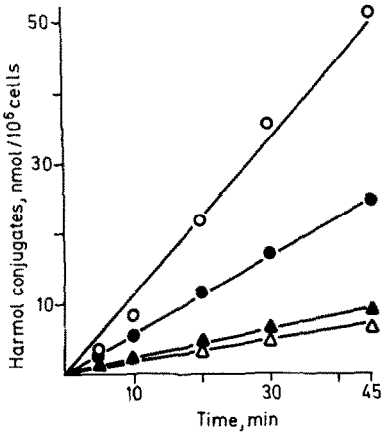

Fig. 2. Time course of harmol conjugation in the presence and absence of 10 mM ethanol in hepatocytes from control rat. Incubations were performed as described in "Materials and Methods". Glucuronides (○—○), and sulphate conjugates (△—△), in the absence of 10 mM ethanol. Glucuronides (●—●), and sulphate conjugates (▲—▲) in the presence of 10 mM ethanol. Values are given as means of 2 different hepatocyte preparations.

probably due to incomplete inhibition of the ethanol oxidation [23]. Present alone, 4-methylpyrazole did not affect glucuronidation. In contrast to glucuronidation, the sulphation of harmol was not inhibited by ethanol addition even up to a concentration of 100 mM (Fig. 1B).

The rate of glucuronidation and sulphation was linear with time for up to 45 min (Fig. 2) in the absence as well as in the presence of 10 mM ethanol. Again, the glucuronidation was inhibited almost 50 per cent and the sulphation was slightly stimulated (Fig. 2).

The effect of ethanol on the conjugation of other substrates was also investigated. As can be seen in Table 1, conjugation of 4-methylumbelliferone, 2-naphthol and phenolphthalein was inhibited in the presence of 10 mM ethanol. Conjugation of phenolphthalein was most sensitive exhibiting an almost

Table 1. Effect of acute ethanol administration on the conjugation of various drug substrates in hepatocytes isolated from control rats

	% of control activity* 10 mM ETOH	
	−MP	+MP
4-Methylumbeniferone (200 μM)	64.3 ± 3.6	93.9 ± 3.2
2-Naphthol (200 μM)	71.0 ± 6.1	105.7 ± 1.2
Phenolphthalein (200 μM)	56.1 ± 6.2	94.6 ± 11.2

Values are means ± S.E.M. of 3–4 different hepatocyte preparations. 10 mM ethanol was incubated in the absence and presence of 1 mM 4-methylpyrazole (MP) for 20 min as described in "Materials and Methods".

* Control activities were for:
4-Methylumbelliferone: 2.04 nmol/10^6 cells/min.
2-Naphthol : 1.18 nmol/10^6 cells/min.
Phenolphthalein : 1.07 nmol/10^6 cells/min.

Table 2. Effect of different agents on the reduction state of NAD(H) in isolated hepatocytes

Additions	% NAD(H) reduced
None	12.0 ± 1.9
Ethanol, 10 mM	28.4 ± 1.1
Ethanol, 10 mM + 4-Methylpyrazole, 1 mM	12.7 ± 0.5
Lactate, 5 mM	21.4 ± 0.03
Sorbitol, 2 mM	22.1 ± 0.9

Incubations were performed in the presence of 200 μM harmol as described in "Materials and Methods". Values represent means ± S.E.M. of 2–3 different hepatocyte preparations.

50 per cent inhibition. The addition of 4-methylpyrazole again reversed the inhibitory effect. It should also be added that 4-methylumbelliferone and 2-naphthol are conjugated with sulphate 22 and 37 per cent, respectively, at the substrate concentration used [24] while phenolphthalein is to more than 90 per cent conjugated with glucuronic acid. Since we measured total conjugation the inhibitory effect of ethanol on the glucuronidation of especially 2-naphthol and 4-methylumbelliferone would be even greater than estimated from total conjugation.

During the alcohol dehydrogenase catalyzed ethanol oxidation the intracellular NADH/NAD^+ ratio is increased [25]. This was true also under our incubation conditions where the presence of ethanol increased the NADH concentration from 12 to 28 per cent (Table 2). In the presence of ethanol + 4-methylpyrazole the NADH level returned to normal. Lactate and sorbitol also increased the NADH/NAD^+ ratio but not to the same extent as ethanol (Table 2). This increase is probably due to the NAD^+ dependent reactions catalyzed by lactate dehydrogenase and sorbitol dehydrogenase.

In this experiment total cellular nicotinamide nucleotide levels were measured. Grundin [9] has shown an even greater increase in the cytoplasmic NADH/NAD^+ ratio by measuring the lactate/pyruvate ratio in the presence of ethanol. This is probably also true for both the lactate and sorbitol catalyzed increase in NADH/NAD^+ ratio, both lactate dehydrogenase and sorbitol dehydrogenase being soluble enzymes.

When investigating the effect of lactate and sorbitol on the conjugation of harmol, a significant inhibition was seen with both substances on glucuronidation, whereas sulphation was unaffected (Fig. 3). The inhibition, which was about 25 per cent, was maximal at 0.5 mM sorbitol and 2 mM lactate.

The intracellular level of UDPGlcUA increased with time in the isolated hepatocytes if no substrate was added to the incubation medium (Fig. 4).

If one assumes a 65 per cent recovery (according to Bock and White [18]), and that harmol is conjugated with all UDPGlcUA present in the added extract (see Materials and Methods) in the presence of microsomes, we obtain a concentration of 1.14 nmol UDPGlcUA per 10^6 cells at the start of incubation

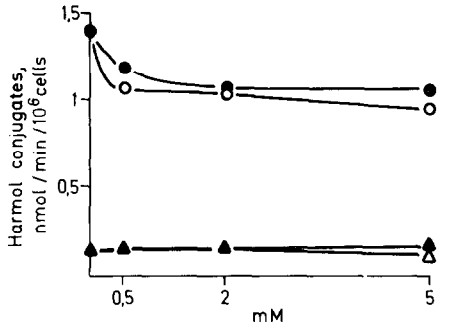

Fig. 3. Effect of lactate (filled symbols) and sorbitol (open symbols) on the conjugation of harmol in hepatocytes isolated from control rat. Incubations were performed as described in "Materials and Methods". ●—●, ○—○, Glucuronides: ▲—▲, △—△, Sulphate conjugates. One typical experiment of three.

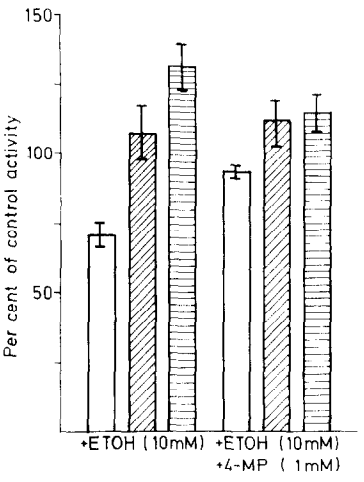

Fig. 5. Effect of ethanol (ETOH) and 4-methylpyrazole (4-MP) on the metabolism of harmine in hepatocytes from phenobarbital treated rats. Incubations were performed as described in "Materials and Methods". ▭, Glucuronides; ▨, Sulphate conjugates; ▤, Harmol. Bars represent means and ranges of two different hepatocyte preparations. The rates expressed as 100 per cent activity were for: glucuronidation, 21.8 nmol/10^6 cells/20 min; sulphate conjugation, 8.6 nmol/10^6 cells/20 min; harmol, 10.6 nmol/10^6 cells/20 min.

reaching 2.18 nmol after 30 min. The concentration of UDPGlcUA reached after 30 min incubation is in the same order of magnitude as what has been found in the isolated perfused liver [18]. One gram of liver represents about 120 × 10^6 cells [26] and this gives a concentration of about 0.26 μmol UDPGlcUA per gram liver.

In the presence of ethanol (12 mM) the rate of increase of UDPGlcUA was significantly inhibited (Fig. 4).

When a substrate undergoing glucuronidation, in this case 4-methylumbelliferone, was present during incubation, there was an immediate drop in the UDPGlcUA level, which then slowly increased with time (Fig. 4). The glucuronidation of 4-methylumbelliferone is linear for at least 30 min with a rate of 1.7 nmol/min/10^6 cells [24]. If one compares the rate of increase of UDPGlcUA calculated from above (0.035 nmol/min/10^6 cells) and the rate of glucuronidation of 4-methylumbelliferone, the glucuronidation rate is very much higher. An explanation of this is that the rate of UDPGlcUA synthesis in the absence of substrate is far from maximal. Not unless UDPGlcUA is used for conjugation is the rate of synthesis accelerated.

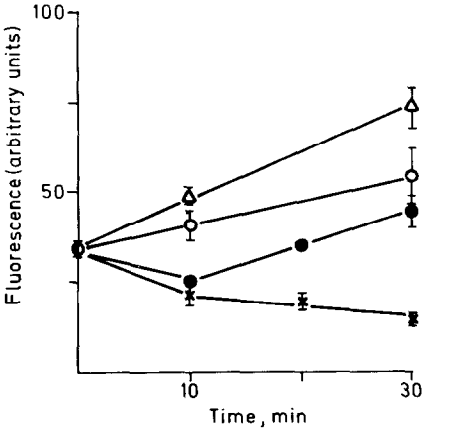

Fig. 4. Effect of ethanol, 4-methylumbelliferone and 4-methylumbelliferone + ethanol on the level of UDP-glucuronic acid in isolated hepatocytes from control rats. Incubations were performed as described in "Materials and Methods". △—△, Control; ○—○, Ethanol, 12 mM; ●—●, 4-methylumbelliferone, 200 μM; × —×, 4-methylumbelliferone, 200 μM, and ethanol, 12 mM. Values are means + S.E.M. of 2–4 different hepatocyte preparations.

Compared with 4-methylumbelliferone present alone, addition of ethanol together with 4-methylumbelliferone further decreased the intracellular level of UDPGlcUA (Fig. 4).

The metabolism of harmine involves an oxidation reaction, catalyzed by cytochrome P-450, to form harmol which is then conjugated with sulphate and glucuronic acid [27]. In control cells the formed harmol is conjugated to form mainly sulphate conjugates [24], and only limited amounts of glucuronides are formed. In these cells addition of ethanol did not alter the conjugation pattern. However, in cells from phenobarbital treated rats where the oxidation rate is increased more than five times and thereby also the amount of glucuronide [24], the addition of ethanol had a significant effect (Fig. 5). The amount of ethanol (10 mM) used did not inhibit the oxidation reaction but the glucuronide conjugation was decreased about 30 per cent with a concomitant increase of the free harmol and also a minor increase in the amount of sulphate conjugate. In the presence of 4-methylpyrazole the effect was reversed, even if not completely (Fig. 5).

DISCUSSION

The results presented in this paper clearly demonstrate the inhibitory effect of low concentrations of ethanol on the glucuronidation of a variety of substances in isolated hepatocytes. This inhibition of glucuronidation was not due to a direct interaction

of ethanol with UDPglucuronosyltransferase but resulted from the alcohol dehydrogenase-dependent oxidation of ethanol. This is evident from both the fact that the ethanol effect was maximal at as low ethanol concentrations as 10 mM and not further increased even at 100 mM and that the inhibition was reversed by 4-methylpyrazole. It is also of interest that the saturating concentration for alcohol dehydrogenase-dependent ethanol oxidation in the hepatocytes is about 6 mM [9]. This concentration is in the same range as that causing maximal inhibitory effect on the glucuronidation. In addition, at these low concentrations, ethanol has little or no effect on glucuronidation in microsomes [6, 13, 15].

During alcohol dehydrogenase catalyzed oxidation of ethanol the NADH/NAD^+ ratio is shifted to the reduced state [25]. This change in redox state has been suggested, at least partly, to be responsible for the adverse effect of ethanol on hepatic metabolism [28]. Our results would also tend to suggest that the increase in NADH/NAD^+ ratio is responsible for the inhibition of glucuronidation and that the effect is not on the conjugation *per se* but on the synthesis of UDPGlcUA.

This suggestion is based on the following facts. The products of ethanol oxidation, acetaldehyde and acetate, did not have any significant effect on the glucuronidation up to 1 mM concentration ([15], and unpublished observation). The rate of glucuronidation was linear even in the presence of ethanol. This also indicates that no accumulated product is responsible for the inhibition (cf. Fig. 2).

Substrates like lactate and sorbitol that also increase the NADH/NAD^+ ratio [29, 30, Table 2] were inhibitory. This inhibition was specific for glucuronidation and showed a similar pattern as the ethanol induced inhibition. It was maximal at rather low concentrations of both lactate and sorbitol and did not increase with increasing concentrations. In addition these substances did not inhibit glucuronidation in microsomes at this concentration (unpublished results).

The synthesis of UDPGlcUA in the hepatocytes did decrease in the presence of ethanol. There was a clear difference between the cellular UDPGlcUA level both in the presence and absence of a substrate undergoing conjugation (cf. Fig. 4). UDPGlcUA is synthesized from glucose and UTP via the formation of UDPglucose which undergoes a subsequent dehydrogenation catalyzed by UDPglucose dehydrogenase. This enzyme is, like alcohol dehydrogenase, NAD^+-dependent and during the course of the reaction 2 molecules of NAD^+ are reduced for each UDPglucose molecule being oxidized (cf. [31]). The proposed inhibition of the synthesis of UDPGlcUA due to the increase in NADH/NAD^+ ratio would then probably result either from limitation of the NAD^+ available for UDPglucose dehydrogenase or rather to a competition between this enzyme and alcohol dehydrogenase for the available NAD^+. This would also suggest that alcohol dehydrogenase has higher affinity for NAD^+ than UDPglucose dehydrogenase. Another possible explanation would be direct inhibition of UDPglucose dehydrogenase by NADH due to the increased cytoplasmic NADH concentration. In fact this enzyme has been shown to be inhibited by NADH [32]. It is not clear, however, whether the rate of the UDPglucose dehydrogenase catalyzed reaction is determined *in vivo* by the NADH/NAD^+ ratio or by the actual concentration of NAD^+ and NADH.

Whether or not the inhibition of glucuronidation observed in isolated hepatocytes has any physiological significance remains to be established. The fact is, however, that the concentrations of ethanol used to obtain maximal effect is well within physiological range. If the effects have physiological significance acute ethanol consumption could alter not only the elimination of foreign compounds but also of endogenous substrates like bilirubine and steroids (cf. [33, 34]).

The inhibitory effect of ethanol on the cytochrome P-450 dependent oxidation reaction have long been established. It is, however, questionable whether this inhibitory action has any significance *in vivo* since rather high concentration of ethanol is needed to obtain inhibition. We found in the hepatocytes no effect on the oxidation reaction of harmine at the concentrations of ethanol used, while when hepatocytes are isolated from phenobarbital treated rats, an inhibition of glucuronidation was observed (cf. Fig. 5). It is notable that no effect on the glucuronide formation in harmine metabolism was seen when control cells were used. These cells have limited oxidation rate and since the formed harmol preferentially conjugates with sulphate, the glucuronidation is only about 20 per cent of maximal [24]. This is of interest since the pattern might be true for a number of drugs and other foreign compounds and in these cases no effect of ethanol on the metabolite pattern would be expected *in vivo*.

In conclusion this study has shown that the interaction between glucuronidation and ethanol is most likely due to an inhibited UDPGlcUA synthesis due to the increased NADH/NAD^+ ratio. The effect has been demonstrated with several substrates and is specific for glucuronidation, while sulphation is unaffected.

Acknowledgements—We wish to thank Ms. Margareta Berggren for excellent technical assistance, Dr. Dean P. Jones for valuable discussions and for correcting the English, and Prof. Sten Orrenius for valuable discussions. This study was supported by grants from the Swedish Medical Research Council (proj. no. 03A-2471 and 03X-2471) and by funds from Karolinska Institutet.

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