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DIMINISHED RATES OF GLUCURONIDATION AND SULFATION IN PERFUSED RAT LIVER AFTER CHRONIC ETHANOL ADMINISTRATION*

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Abstract—Rates of glucuronidation and sulfation of 7-hydroxycoumarin were studied in perfused livers from normal chow-fed rats, or in livers from rats that had been fed liquid control or ethanol-containing diets. During infusion of 100 μM 7-hydroxycoumarin, rates of glucuronidation were similar in livers from chow-fed or control diet rats, but were 34% less in livers from ethanol-fed rats. These rates of glucuronidation in perfused livers could not be explained by changes of UDP-glucuronyltransferase activity, which was highest in hepatic microsomes from ethanol-treated rats and lowest in microsomes from chow-fed rats. The low rates of glucuronidation in livers from ethanol-treated rats were correlated with low hepatic concentrations of UDP-glucuronic acid, which were less than 70% of the levels measured in the other treatment groups. However, the diminished UDP-glucuronic acid levels could not be explained by alterations in adenine nucleotides, NAD^+/NADH ratios, glycogen, UDP-glucose, or activity of UDP-glucose dehydrogenase. Rates of sulfation declined during prolonged 7-hydroxycoumarin infusion in livers from ethanol-treated rats, but not in livers from rats that had received the control diet. Similarly, hepatic concentrations of adenosine-3′-phosphate 5′-sulfatophosphate (PAPS) also decreased with time only in livers from ethanol-treated rats. Thus, chronic ethanol feeding impairs glucuronidation and sulfation in perfused livers as a result of diminished availability of the required cofactors for these conjugation pathways.

The induction of hepatic mixed-function oxidase enzyme activities after chronic exposure to ethanol is well documented. For example, 12–14 days of ethanol consumption has been shown to increase pentobarbital and benzo[*a*]pyrene hydroxylase activities in both rats and humans [1]. Many subsequent studies have shown that these increases in drug-metabolizing activities are correlated with increases in the hepatic concentration of cytochrome P-450 [2], and more recently that chronic ethanol consumption induces a unique form of cytochrome P-450 [3]. In contrast, effects of ethanol on the conjugation of drugs and drug metabolites have received much less attention. Ethanol has been shown to induce bilirubin UDP-glucuronyltransferase activity in the liver of rats and man [4]. Other studies have shown that *p*-nitrophenyl UDP-glucuronyltransferase activity is induced by ethanol in rabbit liver [5] but not in rat liver [6]. In spite of occasional reports of increased glucuronyltransferase activity following ethanol administration, Dutton [7] has concluded that ethanol probably does not induce this enzyme system to any great extent.

In intact cells, rates of conjugation are regulated by both transferase activity and the availability of cofactors [8, 9]. For this reason, perfused rat livers were utilized in these studies to examine the effects of chronic ethanol exposure on rates of glucuronidation and sulfation of the model substrate 7-hydroxycoumarin. The data indicate that chronic ethanol consumption decreased maximal rates of glucuronidation and sulfation in perfused livers, and that this effect was related to diminished availability of cofactors and not to changes in the activity of the transferase enzymes.

METHODS

Animals. Female Sprague–Dawley rats (Sasco, Omaha) weighing 140–150 g were placed on nutritionally adequate liquid diets (Bioserve, Inc.) containing ethanol and fat as 36 and 35% of total calories respectively [10]. Pair-fed controls received liquid diets containing isocaloric carbohydrates in place of ethanol. The diets were given at 9:00 a.m. daily for 14–16 days prior to experiments and were also offered in the mornings that the rats were used in perfusion experiments. Untreated rats received Wayne Lab-Blox and tap water *ad lib.*

Liver perfusion. Liver perfusion was carried out in a nonrecirculating system as described previously [11] with the modification that oxygenation of perfusate was achieved with fritted gas dispersion tubes. Livers were perfused with Krebs–Henseleit bicarbonate buffer [12] which was heated to 37° and saturated with an oxygen–carbon dioxide mixture (95:5). Effluent from the liver was pumped via a cannula placed in the vena cava past a Teflon-shielded oxygen electrode for continuous measurement of venous oxygen tension. Rates of oxygen

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consumption were calculated from the liver weight, flow rate and the arterio-venous oxygen concentration differences and were used to assess tissue viability.

Measurement of rates of 7-hydroxycoumarin conjugation. 7-Hydroxycoumarin was dissolved in the perfusion buffer and infused into livers at the final concentrations shown in the text and figure legends. Samples of effluent perfusate (0.5 ml) were mixed with 2.0 ml of 0.1 M Tris buffer, pH 7.4, or Tris buffer which contained 20 units of β-glucuronidase, highly purified from *Escherichia coli* (Sigma Type VIII), or 0.2 units of sulfatase activity, highly purified from *Aerobacter aerogenes* (Sigma Type VI). More than 98% of the conjugates were hydrolyzed during a 90-min incubation at room temperature. To confirm the specificity of these enzymes, the sulfatase-containing buffer was incubated with glucuronide conjugates of *p*-nitrophenol and 4-methylumbelliferone, and the glucuronidase-containing buffer was incubated with sulfate conjugates of these compounds. No hydrolysis of glucuronide conjugates occurred during incubation with sulfatase, and the glucuronidase did not hydrolyze the sulfate ester conjugates. Furthermore, when glucaro-1,4-lactone (60 μM) was added to samples of perfusate, the glucuronidase activity was abolished, but sulfatase activity was unaffected.

The fluorescence of 7-hydroxycoumarin was measured with excitation and emission wavelengths of 380 and 490 nm respectively. Concentrations of glucuronide and sulfate conjugates in perfusate were determined by differences of 7-hydroxycoumarin measured in samples that were enzymatically hydrolyzed or non-hydrolyzed. Rates of conjugation were calculated from the concentration of conjugate, the flow rate, and the liver wet weight.

Measurement of enzyme activities. UDP-glucuronyltransferase (EC 2.4.1.17) activity was measured in hepatic microsomes that had been isolated by differential centrifugation. The microsomes were washed once and resuspended in 0.15 M KCl. Incubations were performed in 25-ml Erlenmeyer flasks with 2 ml of 0.05 M phosphate buffer, pH 7.0, containing 3 mM UDP-glucuronic acid, 1 mM $MgCl_2$, 0.02% bovine serum albumin, microsomes (1–2 mg/ml), 0.05% Triton X-100 (where indicated) and either *p*-nitrophenol or 7-hydroxycoumarin (aglycone concentration of 1 mM). Incubations were initiated by the addition of UDP-glucuronic acid and were terminated after 20 min by the addition of 0.5 ml of 0.6 N perchloric acid. The precipitated proteins were removed by centrifugation. *p*-Nitrophenol remaining was determined by diluting 0.5 ml of the supernatant fraction with 2.0 ml of 1.6 M glycine buffer, pH 10.3, and reading absorbance at 436 nm ($E_{436} = 7.11\ mM^{-1}\ cm^{-1}$). 7-Hydroxycoumarin remaining was determined by diluting 0.1 ml of the supernatant fraction with 1.9 ml of 0.5 M Tris buffer, pH 7.4, and measuring fluorescence as described above for perfusate.

Phenol sulfotransferase activity (EC 2.8.2.1) was measured according to the methods of Gregory [13], in which the enzyme-catalyzed transfer of sulfate from *p*-nitrophenylsulfate to phenol is measured spectrophotometrically. Hepatic UDP-glucose dehydrogenase activity (EC 1.1.1.22) was assayed by the procedures of Sivaswami *et al.* [14].

Protein concentrations in tissue preparations for the various enzymatic assays were measured by the biuret reaction [15]. Statistical analysis of paired observations was performed by Student's *t*-test, and statistical comparisons among three treatment groups were conducted by analysis of variance.

Measurement of intracellular intermediates. Metabolites were measured in extracts of livers that had been freeze-clamped with tongs chilled in liquid N_2 [16]. The livers were stored at −80° until used for assays. Glycogen was isolated from liver by the procedure of Hassid and Abraham [17], the glucosyl units were hydrolyzed in 1 N HCl, and glucose was measured enzymatically [18]. Perchloric acid extracts of liver were prepared for the enzymatic analysis of adenine nucleotides, lactate and pyruvate [18].

UDP-glucose and UDP-glucuronic acid were measured by a modification of the technique of Wong and Sourkes [19]. Briefly, 0.05-ml aliquots of aqueous liver extracts were added to each of two sets of 50 ml extraction tubes which contained either 0.05 ml of 0.05 M Tris buffer, pH 8.1 (Set 1), or 0.05 ml of the Tris buffer which also contained 0.002 units of UDP-glucose dehydrogenase (Boehringer Mannheim), 4 mM NAD^+, and 4 mM $MgCl_2$ (Set 2). The tubes were incubated at 37° for 60 min, and then 0.1 ml of 0.1 M phosphate buffer, pH 7.0, containing 0.4 mM 4-methylumbelliferone and 0.1% Triton X-100 was added. Finally, 0.1 ml of guinea pig microsomes, suspended in 0.15 M KCl (10 mg protein/ml), was added as a source of glucuronyltransferase which is low in pyrophosphatase activity [20]. After an additional 60 min of incubation at 37°, the reactions were terminated by the addition of 5 ml of 0.05 M sodium acetate, pH 5.0, and the contents of the tubes were extracted three times with 5 ml of $CHCl_3$ to remove unreacted 4-methylumbelliferone. A 0.5-ml aliquot of the final aqueous phase was incubated with 1.5 ml of 0.1 M Tris buffer, pH 7.4, containing 20 units of purified β-glucuronidase (Sigma, Type VIII). After 30 min, 1.0 ml of 1.6 M glycine buffer, pH 10.3, was added, and the fluorescence was measured utilizing excitation and emission wavelengths of 344 and 445 nm respectively. 4-Methylumbelliferone glucuronide standards were carried through the extraction procedure for quantitation of the conjugate formed from tissue extracts. UDP-glucose concentrations were calculated from differences in 4-methylumbelliferone glucuronide formed between the two sets of tubes. Recovery (which averaged approximately 80%) was assessed from internal standards of UDP-glucose and UDP-glucuronic acid added to tissue extracts.

Adenosine-3′-phosphate 5′-sulfatophosphate (PAPS) was measured in boiled hepatic extracts essentially by the technique of Wong and Yeo [21], with the modification that 4-methylumbelliferone (8 mM) was utilized as the fluorescent aglycone instead of harmol. After formation of 4-methylumbelliferone sulfate from PAPS in the extracts, the unreacted 4-methylumbelliferone was extracted in chloroform and the aqueous fractions were incubated with 0.25 units of arylsulfatase, Type VI (Sigma). The extraction procedure and conditions for

measurement of 4-methylumbelliferone fluorescence were as described above for the UDP-glucuronic acid measurements.

RESULTS

Concentration-dependent conjugation of 7-hydroxycoumarin in perfused rat liver. 7-Hydroxycoumarin was infused into livers at concentrations of 10, 40 and 100 μM to study the concentration-dependent formation of glucuronide and sulfate conjugates (Fig. 1). The formation of sulfate conjugates in livers from untreated rats reached near-maximal rates with the infusion of 10 μM 7-hydroxycoumarin, which is characteristic of this high-affinity, easily saturated pathway [8]. In contrast, the formation of glucuronide conjugates increased as the concentration of 7-hydroxycoumarin was increased (Fig. 1). The infusion of 7-hydroxycoumarin at these concentrations caused moderate increases in hepatic respiration (10–30 μmoles O_2/g liver/hr, not shown). However, infusion of 7-hydroxycoumarin at concentrations greater than 100 μM frequently caused inhibition of respiration. To avoid complications of drug toxicity, 100 μM 7-hydroxycoumarin was the highest concentration subsequently studied in perfusion experiments.

Rates of formation of glucuronide and sulfate conjugates of 7-hydroxycoumarin by perfused rat livers after 2 weeks of liquid control or ethanol diets are compared with values from untreated rats in Table 1. Rates of sulfation in livers from rats receiving the control diet were equal to those in livers from untreated rats at all concentrations of 7-hydroxycoumarin tested; however, rates of glucuronidation in livers from the control diet rats were 40–60% higher than rates from untreated rats at the 10 and 40 μM concentrations.

Rates of conjugation in livers from ethanol-treated rats were comparable to the other groups during infusion of 10 μM 7-hydroxycoumarin. However, with 40 μM 7-hydroxycoumarin, rates of glucu-

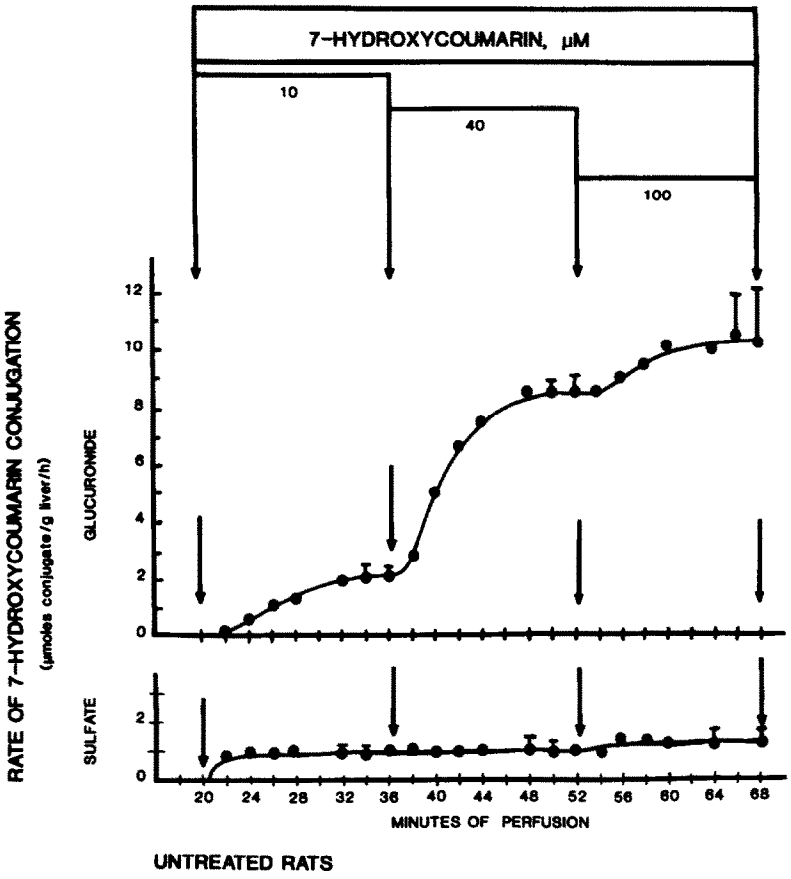

Fig. 1. Concentration-dependent conjugation of 7-hydroxycoumarin in perfused rat livers. After 20 min of perfusion with Krebs–Henseleit bicarbonate buffer, pH 7.4, 7-hydroxycoumarin was infused at concentrations of 10, 40 or 100 μM during the times designated by the horizontal bars and vertical arrows. Samples of effluent perfusate were analyzed for glucuronide and sulfate conjugates as described in Methods, and rates of conjugation were calculated from the concentrations of conjugate, the flow rate and the liver wet weight. The slow approach to steady-state rates of glucuronidation is characteristic of this conjugation pathway in perfusion experiments. Values are averages from three untreated rats. Characteristic S.E. values are shown. Top panel: rates of glucuronidation. Lower panel: rates of sulfation.

Table 1. Concentration-dependent rates of formation of glucuronide and sulfate conjugates of 7-hydroxycoumarin in perfused rat livers

Treatment group	Influent 7-hydroxycoumarin concentration (μM)	Rate of conjugate formation (μmoles/g liver/hr)	
		Glucuronide	Sulfate
Untreated	10	2.09 ± 0.14*	1.07 ± 0.01
	40	7.59 ± 0.56	1.26 ± 0.13
	100	12.33 ± 1.04	1.51 ± 0.24
Control diet	10	3.46 ± 0.22	1.32 ± 0.13
	40	10.25 ± 0.81	1.53 ± 0.16
	100	12.64 ± 0.39	1.88 ± 0.23
Ethanol diet	10	3.02 ± 0.25	1.19 ± 0.18
	40	6.85 ± 0.63*	1.00 ± 0.11*
	100	8.17 ± 0.99*	0.70 ± 0.08*†

Values are means ± S.E. from five to fifteen livers. Rats received liquid diets for 14–16 days prior to experiments. The experimental design is depicted in Fig. 1, and steady-state rates of conjugate formation were calculated as described in Methods.

* Different from corresponding control diet value, $P < 0.05$.

† Different from corresponding ethanol diet value at 10 μM, $P < 0.05$.

ronidation and sulfation were only 66% of the corresponding values of livers from rats that had received the liquid control diet. When the 7-hydroxycoumarin concentration was increased to 100 μM, the rate of glucuronidation in livers from ethanol-treated rats increased over the value at 40 μM, but remained only 66% of rates in the untreated or control diet livers. Rates of sulfation with 100 μM 7-hydroxycoumarin in the ethanol group were less than half of the rates observed in the other groups (Table 1). Moreover, the rate of sulfation at this concentration (0.7 μmole/g/hr) was significantly less than that observed with only 10 μM 7-hydroxycoumarin (1.19 μmoles/g/hr). Rates of conjugation in livers of ethanol-treated rats were not increased by the addition of 10 mM glucose or 5 mM sulfate (data not shown).

Comparative studies were also attempted utilizing *p*-nitrophenol as a substrate for conjugation. However, perfused livers from ethanol-treated rats converted 20–30% of the *p*-nitrophenol into 4-nitrocatechol, which is normally formed in insignificant amounts in perfused livers [22]. Because 4-nitrocatechol competed with *p*-nitrophenol for conjugation pathways, *p*-nitrophenol was not a useful substrate for the study of conjugation reactions in perfused liver after chronic ethanol treatment.

Time-dependent decreases in rates of sulfate conjugation and PAPS levels in livers from ethanol-treated rats. Rates of sulfation in livers from ethanol-treated rats were lowest during infusion of 100 μM 7-hydroxycoumarin (Table 1), which was the final concentration tested in this experimental design (Fig. 1). For this reason, experiments were performed with prolonged infusion of 100 μM 7-hydroxycoumarin to determine whether the substrate concentration or prolonged infusion time caused this decrease in sulfation. Rates of 7-hydroxycoumarin sulfate formation were similar in livers of control and ethanol-treated rats 10 min after infusion of 7-hydroxycoumarin (Table 2). However, rates of sulfation decreased during the next 30 min in livers from

Table 2. Rates of sulfate conjugation and hepatic concentrations of ATP and adenosine-3'-phosphate 5'-sulfatophosphate (PAPS) in perfused rat livers during prolonged infusion of 7-hydroxycoumarin

Treatment group	Minutes after the start of 7-hydroxycoumarin (100 μM) infusion	Rate of sulfate conjugation (μmoles/g/hr)	ATP concn (nmoles/g)	PAPS concn (nmoles/g)
Control diet	10	1.99 ± 0.33	2205 ± 230	19 ± 7
	40	2.35 ± 0.50	2337 ± 78	22 ± 6
Ethanol diet	10	1.30 ± 0.17	1889 ± 147	18 ± 6
	40	0.87 ± 0.20*	2008 ± 128	12 ± 5*

7-Hydroxycoumarin (100 μM) was infused into livers after 20 min of preperfusion with Krebs–Henseleit bicarbonate buffer. After 10 or 40 min of 7-hydroxycoumarin infusion, perfusate samples were collected for measurement of 7-hydroxycoumarin sulfate, and then a portion of liver (0.5 to 0.7 g) was blotted and weighed, and PAPS was measured by a modification of the methods of Wong and Yeo [21] as indicated in Methods. ATP was measured in perchloric acid extracts in separate experiments under identical conditions. Values are means ± S.E. for five to six rats.

* Less than corresponding control value, $P < 0.02$.

Table 3. Effects of liquid ethanol and control diets on hepatic microsomal glucuronyltransferase activities and phenol sulfotransferase activity

Activity measured	Triton X-100 (0.05%)	Untreated rats	Control diet	Ethanol diet
7-Hydroxycoumarin glucuronyltransferase	–	1.0 ± 0.1	2.0 ± 0.1*	2.0 ± 0.5*
(nmoles/min/mg microsomal protein)	+	4.0 ± 0.4	8.0 ± 0.4*	13.0 ± 1.0†
p-Nitrophenyl glucuronyltransferase	–	10.0 ± 0.4	14.0 ± 1.0*	14.0 ± 1.0*
(nmoles/min/mg microsomal protein)	+	15.0 ± 1.0	21.0 ± 1.0*	24.0 ± 1.0†
Phenol sulfotransferase (nmoles/min/mg liver)	N/A‡	0.58 ± 0.06	0.62 ± 0.07	0.72 ± 0.05

Rats received liquid diets for 2 weeks prior to experiments. Glucuronyltransferase activities were measured in washed microsomes as described in Methods. Glucuronyltransferase activity was maximally activated by 0.05% Triton X-100 in all treatment groups. Phenol sulfotransferase was measured according to the methods of Gregory [13]. Values are means ± S.E. for four rats per group.

* $P < 0.05$ with respect to corresponding values from untreated rats.

† $P < 0.05$ with respect to corresponding values from control diet rats.

‡ N/A = non-applicable.

ethanol-treated rats, but not in livers from controls. Similarly, hepatic concentrations of PAPS were identical in the two groups after 10 min of 7-hydroxycoumarin infusion, but values from the ethanol group were decreased to only 55% of the control values after 40 min of 7-hydroxycoumarin infusion (Table 2). Hepatic ATP concentrations in the two groups were not significantly different at either time point. Hepatic phenol sulfotransferase activity was also identical in both groups after 40 min of 7-hydroxycoumarin infusion (not shown).

Effect of chronic ethanol exposure on hepatic glucuronyltransferase and phenol sulfotransferase activities. Hepatic UDP-glucuronyltransferase activity was measured in microsomes from untreated rats and rats fed liquid diets, utilizing the model substrates 7-hydroxycoumarin and *p*-nitrophenol. Triton X-100 was added to solubilize the microsomal membranes and activate enzyme activity [7]. For both aglycones, the lowest glucuronyltransferase activity was measured in microsomes from untreated rats (Table 3). Ethanol feeding increased 7-hydroxycoumarin and *p*-nitrophenyl glucuronyltransferase activities over control diet values in detergent-activated microsomes. Other experiments indicated that ethanol feeding did not change the apparent K_m of microsomal glucuronyltransferases for UDP-glucuronic acid (not shown). Hepatic phenol sulfotransferase activities were comparable in the three treatment groups (Table 3).

Effect of chronic ethanol diets on metabolic intermediates related to conjugation. To determine mechanisms for the observed differences in rates of glucuronidation after chronic ethanol treatment (Table 1), glycogen, UDP-glucose, UDP-glucuronic acid, NAD^+/NADH ratios and UDP-glucose dehydrogenase activity were measured in freeze-clamped livers (Table 4). Rats receiving either liquid diet were found to have hepatic glycogen levels less than half those of untreated rats. UDP-glucose concentrations were similar in the three groups, but levels of UDP-glucuronic acid in livers from ethanol-treated rats were less than 70% of those in the two other groups. In these experiments, no differences were found in cytosolic NAD^+/NADH ratios calculated from measured concentrations of lactate and pyruvate or in UDP-glucose dehydrogenase activities (Table 4).

Effect of pyruvate on lactate production, gluconeogenesis and glucuronidation in perfused rat liver. Because rates of glucuronidation in intact cells are diminished by reductions in cytosolic NAD^+/NADH ratios [24] or depletion of carbohydrate stores [8], pyruvate was infused into livers to attempt to increase rates of glucuronidation. In a liver from an ethanol-treated rat, the infusion of 7-hydroxycoumarin caused a dramatic decrease (85%) in the rate of glucose release into perfusate, and a smaller decrease in the rate of lactate release (Fig. 2). 7-Hydroxycoumarin glucuronide was formed at a rate of 5.2 μmoles/g/hr. The subsequent infusion of pyruvate (1 mM) caused an immediate increase in rates of lactate release of approximately 70 μmoles/g/hr, with a smaller and delayed increase in rates of glucose release. However, pyruvate did not increase rates of glucuronidation in livers of ethanol-treated rats (Fig. 2) or controls (not shown).

DISCUSSION

Induction of glucuronyltransferase activity by chronic ethanol treatment. Liquid nutritionally adequate ethanol-containing diets were devised in order to develop an animal model with an alcohol consumption of clinical relevance [10]. Since ethanol contributes significantly to the caloric intake of the animals (7.1 kcal/g), pair-fed controls are given a diet in which the ethanol calories are replaced by carbohydrates. Although this approach has some advantages over other methods of ethanol treatment, one practical problem is that the controls tend to finish their daily dietary ration within 6–8 hr after feeding, while the animals receiving ethanol feed *ad lib*. The result is that controls go through daily cycles of fasting and refeeding, which may affect various parameters. For example, control diet effects have been noted in studies of hepatic microsomal drug oxidations [25]. For this reason, chow-fed rats were

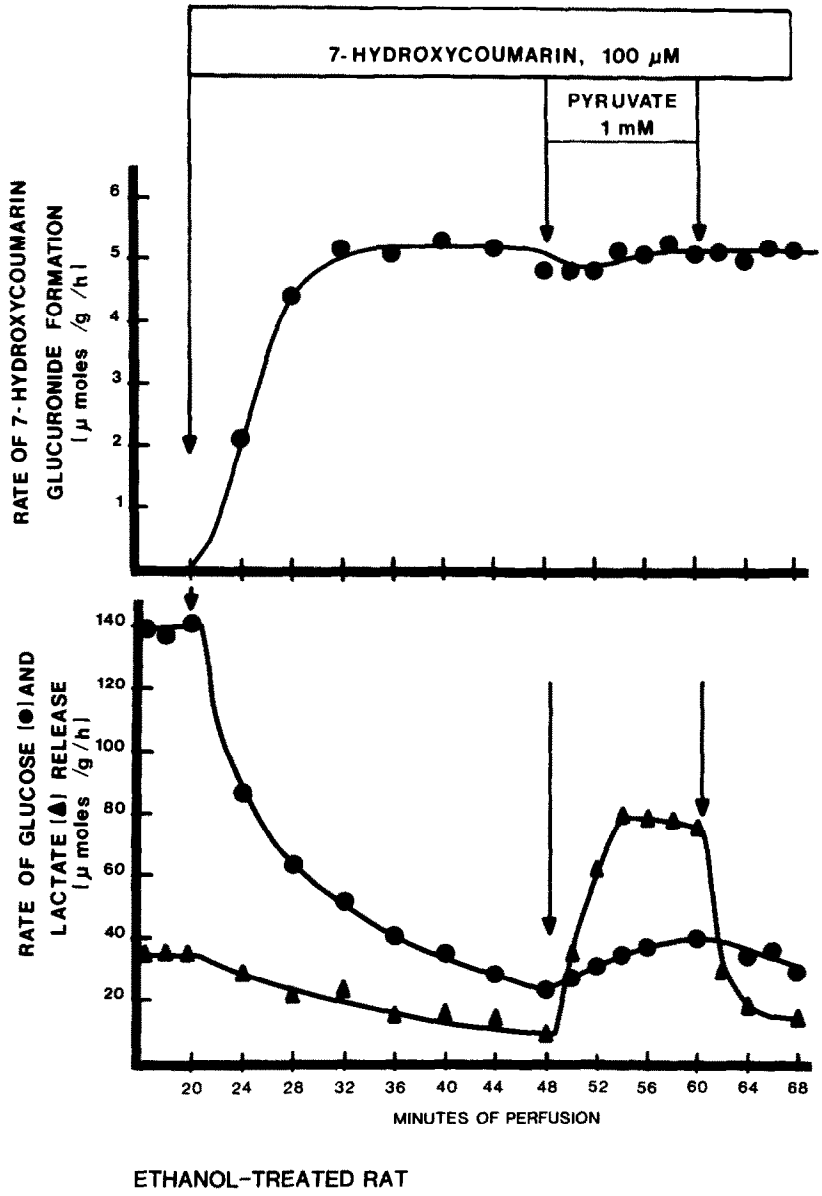

Fig. 2. Effects of pyruvate on rates of lactate, glucose, and 7-hydroxycoumarin glucuronide release from a perfused liver of an ethanol-treated rat. 7-Hydroxycoumarin (100 μM) and sodium pyruvate (1 mM) were infused during the times designated by the horizontal bars and vertical arrows. Glucose, lactate and 7-hydroxycoumarin glucuronide concentrations in perfusate were measured as indicated in Methods, and rates of formation were calculated from the concentration of the metabolites, the flow rate, and liver wet weight.

included in these studies as additional controls. Several differences were noted between rats fed chow or liquid control diets. Rats that received the liquid diet had enhanced microsomal glucuronyltransferase activities (Table 3), which probably explains the tendency for higher rates of glucuronidation at low 7-hydroxycoumarin concentrations (Table 1). Second, hepatic glycogen levels were lower in rats receiving the liquid diet (Table 4), which is possibly related to fasting–refeeding cycles described above. However, in the presence of 100 μM 7-hydroxycoumarin, rates of glucuronidation and sulfation (Table 1) and hepatic concentrations of UDP-glucose and UDP-glucuronic acid (Table 4) were identical in both control groups.

p-Nitrophenyl glucuronyltransferase activity was only slightly increased over control diet values by chronic ethanol treatment (Table 3). This observation is consistent with the lack of induction of this enzyme activity reported by Hietanen *et al.* [6], who administered ethanol as a 15% solution in drinking water. On the other hand, the *p*-nitrophenyl glucuronyltransferase activity in livers from ethanol-treated rats was 50–60% higher than in livers from

Table 4. Effects of liquid ethanol and control diets on glycogen, pyridine nucleotide ratios, UDP-glucose and UDP-glucuronic acid levels in perfused rat livers

Treatment group	Glycogen (μmoles glucosyl units/g)	UDP-glucose (nmoles/g)	UDP-glucuronic acid (nmoles/g)	Cytosolic NAD^+/NADH	UDP-glucose dehydrogenase (nmoles UDP-glucose oxidized/min/mg cytosolic protein)
Untreated rats	82 ± 11	198 ± 17	336 ± 37	878 ± 193	12.86 ± 0.69
Control diet	30 ± 5*	164 ± 12	369 ± 34	832 ± 140	11.76 ± 1.33
Ethanol diet	33 ± 6*	193 ± 36	229 ± 27*†	964 ± 259	12.22 ± 1.48

7-Hydroxycoumarin (100 μM) was infused into livers after 20 min of preperfusion with Krebs-Henseleit bicarbonate buffer, pH 7.4. After 10 min of 7-hydroxycoumarin infusion, the livers were freeze-clamped with tongs cooled in liquid nitrogen, and metabolic intermediates were measured as described in Methods. Cytosolic NAD^+/NADH ratios were calculated from measured concentrations of lactate and pyruvate, assuming an equilibrium constant for lactate dehydrogenase of 0.111 mM [23]. UDP-glucose dehydrogenase was measured by monitoring NADH formation from the metabolism of UDP-glucose [14]. Values are means ± S.E. from five rats per group.

* Different from value in untreated rats, $P < 0.05$.

† Different from values in control diet rats, $P < 0.05$.

untreated rats (Table 3). In rabbits, the administration of 10% ethanol in drinking water increases the activity of *p*-nitrophenyl glucuronyltransferase by 2- to 3-fold [4]. Thus, it can be concluded that induction of glucuronyltransferase activity by ethanol may be species dependent, as well as highly influenced by the mode of ethanol administration.

7-Hydroxycoumarin glucuronyltransferase activity was increased by ethanol when comparisons were made to either control diet rats (30–60% increase) or untreated rats (3-fold increase, Table 3). It is well established that liver contains multiple forms of glucuronyltransferases [26, 27]. The greater induction of 7-hydroxycoumarin glucuronyltransferase activity is probably the result of differing substrate specificities of one or more forms of the enzyme induced by ethanol. However, in spite of high microsomal glucuronyltransferase activity, perfused livers from ethanol-treated rats formed glucuronide conjugates at the lowest rates of the three groups tested (Table 1).

Diminished rates of glucuronidation in perfused livers from ethanol-treated rats, the result of decreased availability of UDP-glucuronic acid. Livers from ethanol-treated rats released 7-hydroxycoumarin glucuronide at rates of only 66% of controls or untreated rats (Table 1). This observation is correlated with a similar decrease in hepatic concentrations of UDP-glucuronic acid, from 335–375 nmoles/g in livers from untreated or control rats, respectively, to 229 nmoles/g in livers from ethanol-treated rats (Table 4). Since the apparent K_m of glucuronyltransferase for UDP-glucuronic acid is about 0.3 mM [7], the observed decrease in UDP-glucuronic acid levels in livers of ethanol-treated rats would be expected to cause a corresponding decrease in rates of glucuronide formation. These observations further demonstrate that rates of glucuronidation in intact cells are highly dependent on levels of UDP-glucuronic acid, and cannot be accurately predicted from glucuronyltransferase activity measured in microsomes [8, 9].

The decreased hepatic levels of UDP-glucuronic acid in livers from ethanol-treated rats cannot be explained by changes in glycogen or UDP-glucose, since concentrations of these intermediates were identical to those in livers of controls (Table 4). Furthermore, infusion of glucose did not increase rates of glucuronidation (Results). A reasonable interpretation of these data is decreased formation of UDP-glucuronic acid by UDP-glucose dehydrogenase. However, the activity of this enzyme was unchanged by ethanol treatment (Table 4). Although UDP-glucose dehydrogenase activity is inhibited by increased levels of intracellular NADH [24], cytosolic NAD^+/NADH ratios were similar in the three groups (Table 4). Furthermore, the possibility of NADH inhibition of this enzyme was disproven by experiments with pyruvate. During infusion of pyruvate, rates of lactate release increased dramatically (Fig. 2) due to oxidation of cytosolic NADH through the action of lactate dehydrogenase, but glucuronidation rates were unchanged during pyruvate infusion.

The diminished availability of UDP-glucuronic acid in livers from ethanol-treated rats is consistent

with at least two possibilities. First, the activity of enzymes which degrade UDP-glucuronic acid (e.g. pyrophosphatase) may be increased, although this effect has not been observed with most microsomal enzyme inducers [28]. Alternatively, UDP-glucose dehydrogenase activity in intact cells may be regulated by other cellular intermediates which are in turn affected by ethanol. It is interesting to note that certain anesthetics have also been shown to decrease hepatic UDP-glucuronic acid levels by an unknown mechanism [29].

Chronic ethanol treatment leading to decreased availability of PAPS and diminished rates of sulfate conjugation. In livers from ethanol-treated rats, rates of sulfation decreased as the 7-hydroxycoumarin concentration was increased from 10 to 100 μM (Table 1). However, this decrease cannot be explained by substrate inhibition, because rates of sulfation were similar in livers of ethanol-treated rats and controls shortly after infusion of 100 μM 7-hydroxycoumarin was started, but subsequently declined only in livers from ethanol-treated rats (Table 2). Thus, the decreasing sulfation rates with higher substrate concentrations shown in Table 1 are the result of prolonged infusion time in this experimental design (Fig. 1).

Sulfate conjugation is a low capacity pathway which is probably limited by the activity of sulfotransferases [8, 30], although the supply of inorganic sulfate may eventually become inadequate in intact animals [31]. Diminished rates of sulfate conjugation in livers from ethanol-treated rats cannot be explained by altered sulfotransferase activity because this activity was unchanged by ethanol treatment in both non-perfused livers (Table 3) and after 40 min of 7-hydroxycoumarin infusion into perfused livers (Results). The observation that rates of sulfation did not decline with time in livers of untreated rats (Fig. 1) or control diet rats (Table 2) also indicates that hepatic sulfotransferases are stable under these experimental conditions. Furthermore, since adequate concentrations of inorganic sulfate are continually supplied to the liver in non-recirculating perfusion systems, the concentration of sulfate should not become rate-limiting for sulfate conjugation. This hypothesis was confirmed by the observations that addition of excess sulfate to the Krebs–Henseleit bicarbonate buffer did not increase rates of sulfate conjugation in livers from ethanol-treated rats (Results) or phenobarbital-treated rats [8]. Koster *et al.* [30] have similarly concluded that sulfation of phenolic drugs in hepatocytes is not limited by the availability of inorganic sulfate.

Low rates of sulfate conjugation in livers from ethanol-treated rats were correlated with low hepatic concentrations of PAPS (Table 2). Thus, it is concluded that the availability of PAPS has become rate-limiting for sulfation under these conditions. However, the reasons for this effect remain obscure. PAPS is synthesized in tissues from inorganic sulfate and ATP by the sequential actions of ATP-sulfurylase and APS-kinase [31]. The average hepatic ATP concentrations in livers from ethanol-treated rats were 86% of the corresponding control values after 10 and 40 min of 7-hydroxycoumarin infusion (Table 2). Although these differences in ATP were not statistically significant, other investigators have reported deleterious effects of ethanol on oxidative phosphorylation [32] and diminished hepatic concentrations of ATP [33] after more prolonged ethanol feeding than was used in these studies. Since hepatic concentrations of PAPS are only about 1% of the concentration of ATP (Table 2), it is perhaps unreasonable to expect that measurable changes in one of these forms of high energy phosphate will be accompanied by changes in the other. Therefore, it is not possible to exclude altered cellular energetics as a mechanism for diminished availability of PAPS in these experiments. Alternatively, ethanol could affect the activity of the enzymes which biosynthesize or degrade PAPS.

In summary, chronic ethanol consumption decreased rates of both glucuronidation and sulfation in perfused livers. The mechanisms for diminished conjugation are not due to altered transferase activities, but are explained by altered biosynthesis and/or degradation of the cofactors UDP-glucuronic acid and adenosine-3′-phosphate 5′-sulfatophosphate. Other phenomena, such as altered competition for conjugation by endogenous compounds or changes in lobular compartmentation of the conjugation reactions, could conceivably be involved in producing these effects. These observations may explain, in part, the recent findings that biliary excretion of glucuronide and sulfate conjugates of acetaminophen is depressed after chronic ethanol feeding [34].

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