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Effects of Ethanol and Sorbitol on Mixed-Function Oxidation in Perfused Rat Livers[1]

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REINKE, L. A., F. C. KAUFFMAN, S. A. BELINSKY AND R. G. THURMAN. *Effects of ethanol and sorbitol on mixed-function oxidation in perfused rat livers.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 289–294, 1983.—Ethanol (20 mM) caused 50–90% inhibition of rates of mixed-function oxidation of p-nitroanisole, 7-ethoxycoumarin and benzo(a)pyrene in perfused rat livers; however, the microsomal metabolism of these substrates was unaltered by low concentrations of ethanol. The metabolism of ethanol was required for this inhibition in the perfused liver. In contrast to ethanol, sorbitol stimulated rates of p-nitroanisole O-demethylation in perfused livers from fasted, phenobarbital-treated rats. Both sorbitol and ethanol infusion decreased the hepatic NAD^+/NADH ratio; however, the $NADP^+$/NADPH ratio was decreased by sorbitol but increased by ethanol. Stimulation of drug metabolism by sorbitol was abolished by pretreatment of fasted rats with 6-aminonicotinamide, an inhibitor of the pentose phosphate shunt. These data indicated that sorbitol stimulated p-nitroanisole metabolism by providing NADPH via the pentose phosphate shunt. The changes in intracellular concentrations of NADPH produced by ethanol and sorbitol correlated directly with changes in hepatic content of citrate and aspartate. These data suggest that inhibition of the citric acid cycle by ethanol decreases the movement of mitochondrial reducing equivalents into the cytosol via substrate shuttle mechanisms.

Inhibition of drug metabolism Ethanol Sorbitol Regulation of mixed-function oxidation Perfused livers

THE role of the mixed-function oxidases in the biotransformation of drugs, toxins and carcinogens has stimulated considerable interest in the regulation of this enzyme system. It is generally assumed that the activity of the mixed-function oxidase enzymes and cytochrome P-450 content are major regulatory factors for rates of xenobiotic transformation in intact cells. Consequently, much attention has been focused on the effects of chemicals, hormones and nutritional state on rates of drug metabolism *in vitro*.

Studies with intact cells have demonstrated that the supply of NADPH may also be a major determinant for rates of mixed-function oxidation in intact cells [11]. In support of this concept, we observed that ethanol inhibited p-nitroanisole O-demethylation [7] whereas sorbitol stimulated this reaction in perfused livers from fasted rats [8]. These opposite effects on mixed-function oxidation were dependent on the metabolism of ethanol and sorbitol which presented an anomaly since both agents increase hepatic levels of NADH. The present study was initiated to compare the effects of sorbitol and ethanol on levels of pyridine nucleotides and intermediates involved in the movement of reducing equivalents from the mitochondrial space into the cytosol. The data indicate that sorbitol increases NADPH supply by providing substrate for the pentose phosphate shunt whereas ethanol inhibits the movement of reducing equivalents of mitochondrial origin into the cytosol.

METHOD

Female Sprague-Dawley rats, 100–200 g, were used in all experiments. Where indicated, rats were treated with sodium phenobarbital (1 mg/ml) in drinking water; liquid diets containing ethanol as 36% of total calories or isocaloric carbohydrates [2]; or 3-methylcholanthrene (20 mg/kg, IP) dissolved in corn oil and given on 3 consecutive days prior to

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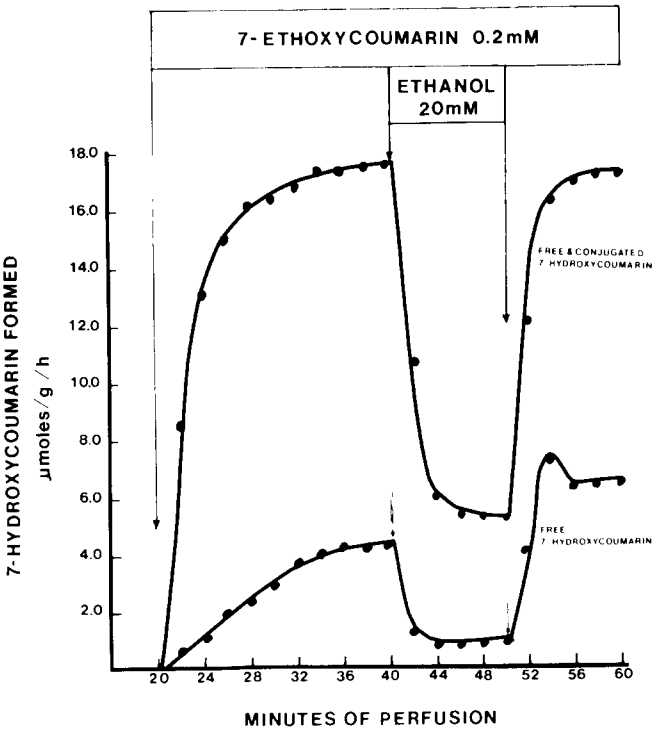

FIG. 1. Inhibition of 7-ethoxycoumarin O-demethylation in a perfused liver from a rat treated with a diet containing ethanol. Samples of perfusate were assayed for 7-hydroxycoumarin either directly (free) or after hydrolysis of glucuronide and sulfate conjugates (free and conjugated) as described in the Method section. Infusion of 7-ethoxycoumarin (0.2 mM) and ethanol (20 mM) are designated by the horizontal bars and vertical arrows.

perfusion experiments. Fasted animals were deprived of food for 24 hours prior to use.

Details of the perfusion technique have been described elsewhere [7,8]. Livers were perfused at 37°C with Krebs-Henseleit bicarbonate buffer, pH 7.4, saturated with an oxygen-carbon dioxide mixture (95:5) in a non-recirculating system. p-Nitroanisole (0.2 mM) and 7-ethoxycoumarin (0.2 mM) were dissolved in Krebs-Henseleit bicarbonate buffer. Benzo(a)pyrene was suspended in a solution of 15% bovine serum albumin and infused into livers at a final concentration of 0.01 mM as described elsewhere [9]. Samples of perfusate (1.0 ml) were incubated with 25 units of sulfatase and 275 units of β-glucuronidase (Sigma) for 90 minutes to hydrolyze glucuronide and sulfate conjugates. p-Nitrophenol formation from p-nitroanisole was measured spectrally at 436 nm [7,8] and 7-hydroxycoumarin was measured fluorimetrically with excitation and emission wavelengths of 380 nm and 490 nm, respectively. Benzo(a)pyrene phenols were measured in samples of effluent perfusate by a modification of the methods of Dehnen *et al.* [3]. Ethanol and sorbitol were dissolved in buffer and infused into the perfusion fluid entering the liver at final concentrations indicated in the text and figure legends.

Perfused livers were freeze-clamped with tongs chilled in liquid nitrogen. Pyridine nucleotides were measured in alkaline extracts of liver by enzymatic cycling techniques [6] and intermediates of the citric acid cycle were measured in $HClO_4$ extracts of liver by methods described elsewhere [4]. Hepatic microsomes were prepared by differential centrifugation, and mixed-function oxidase activities were assayed by methods described previously [7,8].

RESULTS

Effect of Ethanol on Rates of Mixed-Function Oxidation in Perfused Rat Livers

Ethanol inhibited rates of mixed-function oxidation in perfused rat livers under a variety of experimental conditions. For example, when 7-ethoxycoumarin (0.2 mM) was infused into a liver from a rat treated chronically with ethanol [2], the maximal rate of 7-hydroxycoumarin formation was approximately 18 μmoles/g/hr (Fig. 1). Under these conditions, infusion of ethanol (20 mM) inhibited the rate of 7-hydroxycoumarin formation by 70%. This inhibition was reversible when ethanol infusion was terminated (Fig. 1). Similar results were obtained with livers from phenobarbital-treated rats, utilizing p-nitroanisole as the substrate for mixed-function oxidation (Fig. 2A).

Table 1 summarizes the effect of ethanol on rates of mixed-function oxidation of selected substrates in perfused livers under a variety of different experimental conditions. Rates of metabolism in these studies ranged from 0.04 μmoles/g/hr for phenol release from livers of 3-methylcholanthrene-treated rats during benzo(a)pyrene infusion to 14.8 μmoles/g/hr for 7-hydroxycoumarin formation from 7-ethoxycoumarin in perfused livers from rats treated chronically with ethanol. Ethanol (20 mM) inhibited rates of mixed-function oxidation in perfused livers by 50–90% under all experimental conditions (Table 1). In contrast, much higher concentrations of ethanol were required to inhibit mixed-function oxidation in microsomes. For example, 130 mM ethanol was required to inhibit p-nitroanisole metabolism 50% [7], and 500 mM ethanol caused only 15% inhibition of benzo(a)pyrene hydroxylation [9] in microsomal preparations.

The effect of increasing concentrations of ethanol on p-nitroanisole metabolism in livers from fed, phenobarbital-treated rats is depicted in Fig. 3. Progressive inhibition of mixed-function oxidation was observed when the ethanol concentration was increased from 1 to 5 mM. Increasing the ethanol concentration to 20 mM caused a slight further inhibition of p-nitroanisole O-demethylation. When the percentage inhibition by ethanol in microsomes was subtracted from the inhibition in perfused liver (PL-M; Fig. 3), about 50% inhibition of p-nitroanisole O-demethylation in the perfused liver could not be explained by direct effects of ethanol on the drug-metabolizing enzymes. Furthermore, this inhibition by ethanol was half-maximal at 1–2 mM ethanol (Fig. 3). Similar data were obtained with 7-ethoxycoumarin in livers from rats receiving diets containing ethanol (data not shown).

Low concentrations of acetaldehyde (1–2 mM) were as effective as ethanol in inhibiting p-nitroanisole [7] or 7-ethoxycoumarin metabolism (not shown) in perfused livers. t-Butanol (20 mM), a hydrophobic alcohol which is not a substrate for alcohol dehydrogenase, did not inhibit p-nitroanisole metabolism [7].

Effect of Sorbitol on Rates of Mixed-Function Oxidation in Perfused Livers from Fasted Rats

Sorbitol stimulates p-nitroanisole metabolism in perfused

TABLE 1

INHIBITION OF MIXED-FUNCTION OXIDATION IN PERFUSED RAT LIVERS BY ETHANOL

Treatment	Substrate for Mixed-Function Oxidation	Maximal Rate of Product Formation (μmoles/g liver/hr)	% Inhibition by Ethanol (20 mM)
None	7-Ethoxycoumarin	2.9 ± 0.5	49
Ethanol Diet	7-Ethoxycoumarin	14.8 ± 0.9	70
Control Diet	7-Ethoxycoumarin	2.2 ± 0.7	43
None	p-Nitroanisole	2.4 ± 0.7	90
Phenobarbital	p-Nitroanisole	8.9 ± 1.1	78
3-Methylcholanthrene	Benzo(a)pyrene	0.04 ± 0.01	50

Rats were pretreated as described in the Method section. Maximal rates of mixed-function oxidation were calculated from concentrations of the following products measured in effluent perfusate: 7-hydroxycoumarin from 7-ethoxycoumarin (0.2 mM); p-nitrophenol from p-nitroanisole (0.2 mM); Benzo(a)pyrene phenols from benzo(a)pyrene (0.01 mM). The percent inhibition was calculated from changes in steady-state rates of mixed-function oxidation caused by ethanol infusion (see Figs. 1 and 2A). Values are means ± S.E.M.

livers from fasted, but not fed, phenobarbital-treated rats [8]. This stimulation correlated directly with the metabolism of sorbitol detected as an increase in the fluorescence of reduced pyridine nucleotides monitored from the liver surface.

In perfused livers from fasted, phenobarbital-treated rats, sorbitol (2 mM) increased rates of p-nitroanisole O-demethylation by about 3 μmoles/g/hr (Fig. 2B). However, if rats were pretreated with 6-aminonicotinamide, an

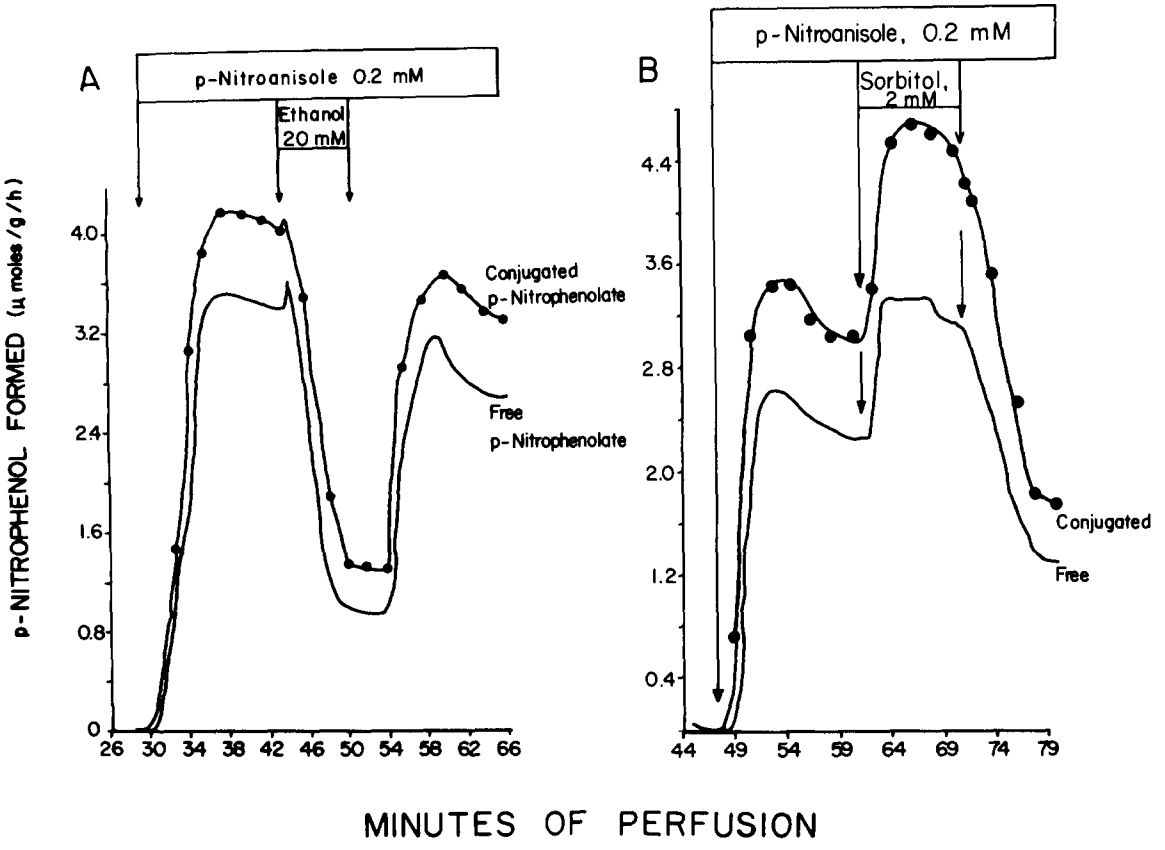

FIG. 2. Effects of ethanol and sorbitol on p-nitroanisole O-demethylation in perfused livers from fasted, phenobarbital-treated rats. Free and conjugated p-nitrophenol were measured as described in the legend to Fig. 1 and in the Method section. Infusions of p-nitroanisole (0.2 mM), ethanol (20 mM, Panel A) and sorbitol (2 mM, Panel B) are designated by the horizontal bars and vertical arrows.

TABLE 2

EFFECTS OF ETHANOL AND SORBITOL ON CELLULAR INTERMEDIATES IN PERFUSED LIVERS FROM PHENOBARBITAL-TREATED RATS

	μmoles/kg wet liver weight		
	Controls (9)	Ethanol (20 mM) (4)	Sorbitol (5 mM) (5)
$NAD^+/NADH$	5.8 ± 1.1	1.4 ± 0.1†	1.0 ± 0.1†
$NADP^+/NADPH$	2.8 ± 0.3	3.9 ± 0.7*	0.5 ± 0.1‡
Citrate	16 ± 3	12 ± 3	22 ± 2†
Malate	33 ± 4	54 ± 12	31 ± 6
Glutamate	681 ± 47	837 ± 108	907 ± 86†
Aspartate	303 ± 22	264 ± 25	504 ± 80†

Livers were perfused with p-nitroanisole (0.2 mM) after 20 minutes of preperfusion with Krebs-Henseleit bicarbonate buffer. Ethanol and sorbitol were introduced after 20 minutes of p-nitroanisole infusion, and all livers were freeze-clamped 8 minutes later. Pyridine nucleotides were measured in alkaline extracts and all other metabolites were measured in acid extracts of tissue as described in METHODS. Values are means ± S.E.M. for the number of livers shown in parenthesis.

*$p=0.07$, †$p<0.05$, ‡$p<0.001$, with respect to controls.

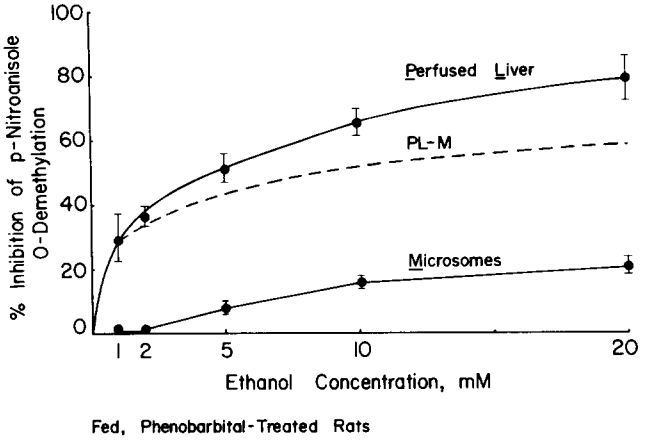

FIG. 3. Effect of ethanol concentration on p-nitroanisole O-demethylase activity in hepatic microsomes and perfused livers from fed, phenobarbital-treated rats. The data points are derived from 4–6 experiments of the design illustrated in Fig. 1. The percent inhibition was calculated from steady-state changes in rates of p-nitroanisole metabolism in perfused livers produced by ethanol. Microsomal data was obtained as described elsewhere [7,8].

inhibitor of the pentose phosphate shunt [5], p-nitroanisole metabolism was not stimulated by sorbitol (not shown). The infusion of sorbitol increased rates of glucose and lactate production by perfused livers from both untreated and 6-aminonicotinamide-treated rats (data not shown).

Effect of Ethanol and Sorbitol on Intracellular Pyridine Nucleotide Content and Other Metabolic Intermediates

Under conditions similar to those shown in Fig. 2, both ethanol and sorbitol decreased hepatic $NAD^+/NADH$ ratios significantly in livers from fasted rats (Table 2). This reduction of NAD^+ was anticipated since sorbitol and ethanol are metabolized predominantly by the NAD^+-requiring enzymes alcohol dehydrogenase and sorbitol dehydrogenase, respectively. In contrast, the effect of ethanol and sorbitol on the $NADP^+$ oxidation:reduction state differed remarkably. Sorbitol infusion caused a large reduction in $NADP^+$ while ethanol caused an oxidation of NADPH (Table 2). Sorbitol increased the hepatic concentrations of citrate, glutamate and aspartate whereas ethanol tended to decrease concentrations of citrate and aspartate.

DISCUSSION

Ethanol Inhibits Mixed-Function Oxidation in Perfused Livers as a Consequence of Its Metabolism

Ethanol (20 mM) caused 50–90% inhibition of rates of mixed-function oxidation in perfused livers, regardless of the induction state of the animals or the rate of biotransformation of the various substrates tested (Table 1). In contrast, inhibitory concentrations of ethanol in microsomal studies were much higher than concentrations required to produce comparable inhibition in intact livers (Fig. 3). Thus, a direct hepatic microsomal interaction [10] is an unlikely mechanism for inhibition of mixed-function oxidation by low concentrations of ethanol in perfused livers.

A number of observations from these and other studies implicate the metabolism of ethanol via alcohol dehydrogenase in the inhibition of mixed-function oxidation. First, concentrations of ethanol required to cause half-maximal inhibition of p-nitroanisole (1–2 mM, Fig. 3) and 7-ethoxycoumarin are near the Km of alcohol dehydrogenase for ethanol. Second, acetaldehyde was also an effective inhibitor of p-nitroanisole [7] and 7-ethoxycoumarin metabolism in perfused rat livers. Third, t-butanol, which is not a substrate for alcohol dehydrogenase, did not inhibit p-nitroanisole metabolism [7]. Finally, ethanol inhibition of p-nitroanisole metabolism was minimal in the presence of 4-methylpyrazole, a potent inhibitor of alcohol dehydrogenase [7]. The complete oxidation of one mole of ethanol to acetate leads to the synthesis of 2 moles of NADH. Thus,

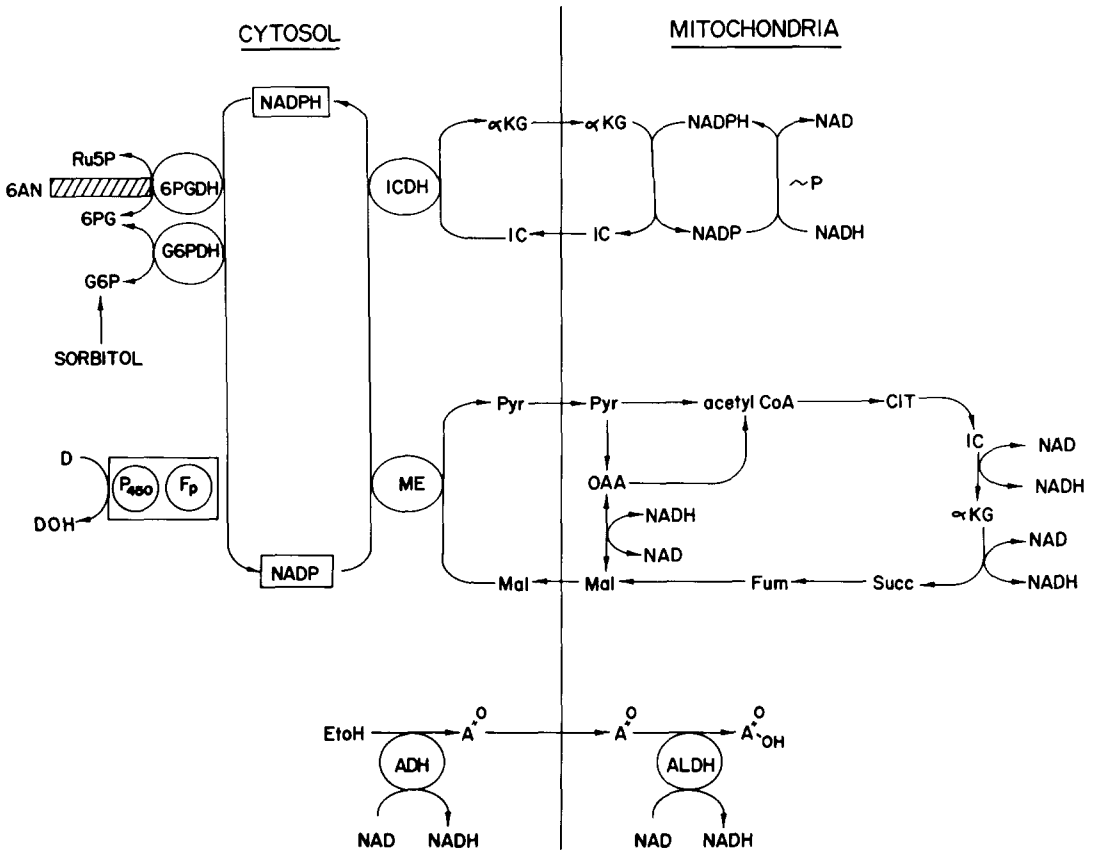

FIG. 4. Metabolic interactions between sorbitol metabolism and NADPH generation. The scheme depicts the metabolism of ethanol and sorbitol by alcohol dehydrogenase (ADH) and sorbitol dehydrogenase. NADPH utilized by the cytochrome P-450-dependent monooxygenases (P450), is subsequently regenerated by isocitrate dehydrogenase (ICDH) and the pentose phosphate shunt enzymes glucose-6-phosphate dehydrogenase (GGPDH) and 6-phosphogluconate dehydrogenase (GPGDH). Abbreviations used are IC, isocitrate; αKG, α-ketoglutarate; G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; R5P, ribulose-5-phosphate; Fp, flavoprotein; 6AN, 6-aminonicotinamide; D, drug; DOH, hydroxylated drug; ME, "malic" enzyme; Pyr, pyruvate; Mal, malate; OAA, oxalacetate; CIT, citrate; Succ, succinate; Fum, fumarate; ALDH, aldehyde dehydrogenase; A^{O}, acetaldehyde; A^{O}_{OH}, acetate.

ethanol and acetaldehyde oxidation cause a reduction in intracellular NAD^+ as reflected by a decrease in the NAD^+/NADH ratio (Table 2). This elevation in intracellular NADH causes redox inhibition of the citric acid cycle [1] leading to decreased intracellular concentrations of citrate and aspartate [7] (Table 2). Ethanol has also been shown to decrease levels of isocitrate and α-ketoglutarate in perfused rat liver [7]. Thus, the oxidation of NADPH observed in the presence of ethanol (Table 2) is likely explained by diminished $NADP^+$-dependent isocitrate dehydrogenase activity subsequent to inhibition of the citric acid cycle (Fig. 4). This hypothesis is supported by the observation that aspartate, which provides oxalacetate for the citric acid cycle, reversed partially the inhibition of mixed-function oxidation by ethanol [7]. Thus, ethanol inhibits mixed-function oxidation by diminishing the flow of NADPH derived from mitochondrial oxidations into the cytosol (Fig. 4).

Mechanism for Stimulation of Mixed-Function Oxidation by Sorbitol

Sorbitol infusion into livers from fasted rats stimulated rates of p-nitroanisole metabolism (Fig. 2B) and increased intracellular levels of NADH and NADPH (Table 2). In liver, sorbitol is metabolized primarily to fructose by an NAD^+-requiring sorbitol dehydrogenase, which results in increased hepatic contents of NADH. Experiments with 6-aminonicotinamide demonstrated the mechanism for increased levels of NADPH. 6-Aminonicotinamide is converted by a variety of tissues into 6-amino-$NADP^+$, a potent inhibitor of 6-phosphogluconate dehydrogenase [5]. The failure of sorbitol to stimulate p-nitroanisole O-demethylation after 6-aminonicotinamide pretreatment cannot be explained by decreased metabolism of the carbohydrate since 6-aminonicotinamide does not prevent the conversion of sorbitol to lactate and glucose (see Results, above). Thus, we conclude that sorbitol stimulates mixed-function oxidation by the following sequence of events: First, sorbitol is converted into fructose by the NAD^+-dependent sorbitol dehydrogenase; second, fructose is phosphorylated and converted into glucose-6-phosphate; finally, the metabolism of glucose-6-phosphate and 6-phosphogluconate via the pentose phosphate shunt provides NADPH for mixed-function oxidation (Fig. 4).

NADH-mediated inhibition of the citric acid cycle is intimately involved in the mechanism of inhibition of drug metabolism by ethanol. Although sorbitol caused similar changes in the $NAD^+/NADH$ ratio (Table 2) as ethanol, it did not inhibit drug metabolism. A possible explanation is that stimulation of the pentose phosphate shunt by sorbitol offsets inhibition of the generation of mitochondrial NADPH. However, because sorbitol did not inhibit drug metabolism in 6-aminonicotinamide-treated rats, this possibility can be excluded. The lack of inhibition most likely stems from the fact that sorbitol increases the supply of substrate for the citric acid cycle whereas ethanol does not. Thus, like aspartate, sorbitol could increase flux in the citric acid cycle providing intermediates both for NADPH synthesis and substrate shuttle mechanisms (Fig. 4). This hypothesis is supported by the observation that citrate and aspartate were elevated by the infusion of sorbitol (Table 2).

Taken together, these data indicate that mitochondrial citric acid cycle activity may be required for extramitochondrial mixed-function oxidation in a variety of metabolic states.

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