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INTERACTIONS BETWEEN PLASTICIZERS AND FATTY ACID METABOLISM IN THE PERFUSED RAT LIVER AND *IN VIVO*

INHIBITION OF KETOGENESIS BY 2-ETHYLHEXANOL

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Abstract—Rates of ketone body (β-hydroxybutyrate plus acetoacetate) production by perfused livers from starved rats were decreased about 60% from 39 ± 2 to 17 ± 3 μmol/g/hr by 2-ethylhexanol (200 μM), a primary metabolite of the plasticizer diethylhexyl phthalate. Inhibition of ketogenesis by ethylhexanol was dose dependent (half-maximal inhibition occurred with 25 μM) in the presence or absence of 4-methylpyrazole, an inhibitor of alcohol dehydrogenase. Concentrations of β-hydroxbutyrate relative to acetoacetate (B/A) increased in a step-wise manner from 0.32 to 0.75 in the effluent perfusate when ethylhexanol was infused. In contrast, the B/A ratio decreased in parallel with inhibition of ketone body production when alcohol dehydrogenase was inhibited. Pretreatment of rats with phenobarbital, an inducer of ω and ω-1 hydroxylases, diminished inhibition of ketone body production by low (<50 μM) but not high concentrations (>50 μM) of ethylhexanol. Thus, ethylhexanol is oxidized via phenobarbital-inducible pathways to metabolites which do not inhibit ketogenesis. Studies were conducted to determine the site of inhibition of fatty acid oxidation by ethylhexanol. Rates of ketone body production in the presence of oleate (250 μM), which requires transport of the corresponding CoA compound into mitochondria, were reduced from 80 ± 6 to 58 ± 8 μmol/g/hr by ethylhexanol. In contrast, ketone body production from hexanoate, which is activated in the mitochondria, was not affected by ethylhexanol. Basal and oleate-stimulated rates of H_2O_2 production were not affected by ethylhexanol, indicating that peroxisomal β-oxidation was not altered by the compound. Based on these data it is concluded that 2-ethylhexanol inhibits β-oxidation of fatty acids in mitochondria but not in peroxisomes. Treatment of rats with ethylhexanol (0.32 g/kg, i.p.) decreased plasma ketone bodies from 1.6 to 0.8 mM, increased hepatic triglycerides and increased lipid predominantly in periportal regions of the liver lobule. These data indicate that alterations in hepatic fatty acid metabolism in periportal regions of the liver lobule may be early events in peroxisome proliferation.

Phthalic acid esters are widely used as plasticizers, with an annual production of about two million tons in the United States alone [1]. These ubiquitous chemicals have become environmental pollutants and are found in soil, plants and the atmosphere as well as in a variety of foods and milk [1]. Exposure to phthalates also occurs via plastic medical devices such as heart valves, blood transfusion sets and disposable syringes [1]. Patients undergoing hemodialysis for 5 hr can receive up to 150 mg of diethylhexyl phthalate, the most common phthalate plasticizer [2]. Jaeger and Rubin [3] reported the presence of diethylhexyl phthalate in abdominal fat, liver, spleen and lungs of patients receiving large volumes of blood stored in plastic bags, and Hillman *et al.* [4] found phthalate in neonatal heart and gastrointestinal tissues of infants transfused via umbilical catheters.

Peroxisomes have a unique fatty acid metabolizing system [5, 6] where β-oxidation of acyl-CoA compounds generates one mole of H_2O_2 for each mole of acetyl-CoA produced, while mitochondrial β-oxidation produces NADH but not H_2O_2 [6]. In normal liver, peroxisomes are more abundant in pericentral than periportal regions of the liver lobule and the ratio of peroxisomes to mitochondria is approximately 1:5 [7]. This ratio is increased markedly in hepatocytes of rats fed phthalates, which cause dose-dependent increases in liver weight, peroxisomes and peroxisomal enzyme activities (e.g. catalase and fatty acyl-CoA oxidase) [8–11]. The exact mechanism by which these chemicals cause peroxisomal proliferation is not known; however, an increase in transcription of peroxisome-specific RNA occurs [12].

Chronic treatment of animals with agents which cause proliferation of peroxisomes has long been known to alter fatty acid metabolism. Therefore, the purpose of this study was to investigate the effects of acute exposure of the isolated, perfused liver to

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ethylhexanol, a primary metabolite of the plasticizer diethylhexyl phthalate and a known inducer of peroxisomes [13, 14], on mitochondrial and peroxisomal β-oxidation. The data indicate that ethylhexanol inhibits mitochondrial but not peroxisomal β-oxidation in perfused rat livers. Preliminary accounts of this work have appeared elsewhere [15].

MATERIALS AND METHODS

Materials. 2-Ethylhexanol was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Oleate, hexanoate, albumin and osmium tetroxide were purchased from the Sigma Chemical Co. (St Louis, MO). All other chemicals were of reagent grade from standard sources.

Liver perfusion. Female Sprague–Dawley rats (200–250 g) were starved for 24 hr prior to use. Where indicated, rats were given phenobarbital in drinking water (1 g/L) for 7 days prior to being killed to induce ω and ω-1 fatty acid oxidases. Livers were perfused via the portal vein with Krebs–Henseleit bicarbonate buffer (pH 7.4, 37°) saturated with 95% O_2:5% CO_2 as described previously [16]. Ethylhexanol was dissolved in Krebs–Henseleit buffer and was infused into the liver with a precision infusion pump. Oleate and hexanoate were bound to defatted bovine serum albumin (fatty acid:albumin ratio, 4:1) prior to infusion. The final concentration of albumin infused was 0.3%. Acetoacetate and β-hydroxybutyrate in the effluent perfusate or plasma were assayed by standard enzymatic techniques [17], and rates were calculated from concentrations of ketone bodies in the effluent perfusate, the flow rate and the liver wet weight. Concentrations of oxygen in the effluent perfusate were monitored continuously with a Teflon-shielded, Clark-type oxygen electrode. Rates of oxygen uptake were calculated from influent–effluent concentration differences, the flow rate and the liver wet weight, and were used to assess viability of livers.

Measurement of H_2O_2 generation by perfused liver. The steady-state level of catalase–H_2O_2 compound I was determined spectrophotometrically (660–640 nm) through a lobe of the liver employing an air-turbine driven dual wavelength spectrophotometer as described previously [18]. Rates of H_2O_2 generation were quantitated by the method of Oshino *et al.* [19]. Briefly, methanol (26 mM) was infused to decrease the steady-state level of catalase–H_2O_2 maximally. Methanol concentrations were then decreased in a step-wise manner and increases in steady-state amounts of catalase–H_2O_2 were recorded. Rates of H_2O_2 generation were calculated from the concentration of methanol needed to decrease catalase–H_2O_2 by 50%, the amount of catalase heme and kinetic constants for rat liver catalase [19].

Measurement of hepatic lipid content. At 2, 4 or 6 hr after pretreatment with 2-ethylhexanol (0.32 g/kg, i.p.), rats were anesthetized with pentobarbital and livers were perfused to remove blood. Subsequently, 1% paraformaldehyde in Krebs–Henseleit buffer was infused for 8 min. Fixed tissue sections were washed with Krebs–Henseleit buffer and post-fixed in 1% osmium tetroxide–2.5% potassium dichromate. Samples were washed subsequently for 2 hr with tap water and processed for light microscopy [20].

For determination of total lipid and triglycerides, starved rats were given 2-ethylhexanol (0.32 g/kg, i.p.) 100 min before freeze clamping of livers *in situ*. Total lipids were measured gravimetrically in $CHCl_3$:CH_3OH (1:1) extracts of freeze-clamped livers after evaporation of the solvent in a Savant speedvac concentrator. Triglycerides in the lipid residue were measured enzymatically as described elsewhere [21].

Statistical analyses. Statistical comparisons were performed with Student's *t*-test.

RESULTS

Inhibition of hepatic ketogenesis by 2-ethylhexanol in the isolated perfused rat liver. Basal rates of ketone body production by perfused livers from normal, starved rats were about 39 μmol/g/hr (Fig. 1A). Infusion of increasing concentrations of ethylhexanol decreased ketone body production rapidly in a step-wise manner. Half-maximal inhibition was observed at a concentration of about 25 μM ethylhexanol and maximal inhibition of about 60% occurred with 200 μM (Fig. 1A and 2). When ethylhexanol infusion was terminated, rates of ketone body production returned toward basal values (Fig. 1A). 4-Methylpyrazole (80 μM), an inhibitor of alcohol dehydrogenase, did not reverse the inhibition of ketone body production by ethylhexanol (Fig. 1B and 2). Pretreatment of rats with phenobarbital abolished the inhibitory effects of low (<50 μM) concentrations of ethylhexanol and attenuated the effects of higher concentrations.

Concomitantly, ethylhexanol caused a reduction of the mitochondrial NADH redox state (NADH/NAD^+ ratio) reflected by a 2-fold increase in the β-hydroxybutyrate to acetoacetate (B/A) ratio in the effluent perfusate (Fig. 1C). In contrast, the B/A ratio was decreased in parallel with inhibition of fatty acid β-oxidation, an NADH-producing process, in the presence of 4-methylpyrazole.

Effect of ethylhexanol on H_2O_2 generation by perfused rat liver. Rates of hepatic H_2O_2 production can be determined by measuring the destruction of the catalase–H_2O_2 complex by methanol [19]. Experiments were initiated by infusing glycolate (1 mM) to establish maximal saturation of catalase heme with H_2O_2. Infusion of a high concentration of methanol (26 mM) diminished the steady-state level of the catalase–H_2O_2 complex totally (Fig. 3). When methanol concentrations were subsequently decreased in steps, the steady-state level of catalase–H_2O_2 increased in a step-wise manner (Fig. 3). In livers from normal starved rats perfused in a non-recirculating system, basal rates of H_2O_2 production determined in this manner were 8 ± 2 μmol/g/hr (mean ± SE, N = 4). Rates of H_2O_2 production were increased markedly by infusion of oleate (1 mM) to 13 ± 1 μmol/g/hr. However, oleate-stimulated rates of H_2O_2 production were not affected by infusion of ethylhexanol (250 μM; data not shown).

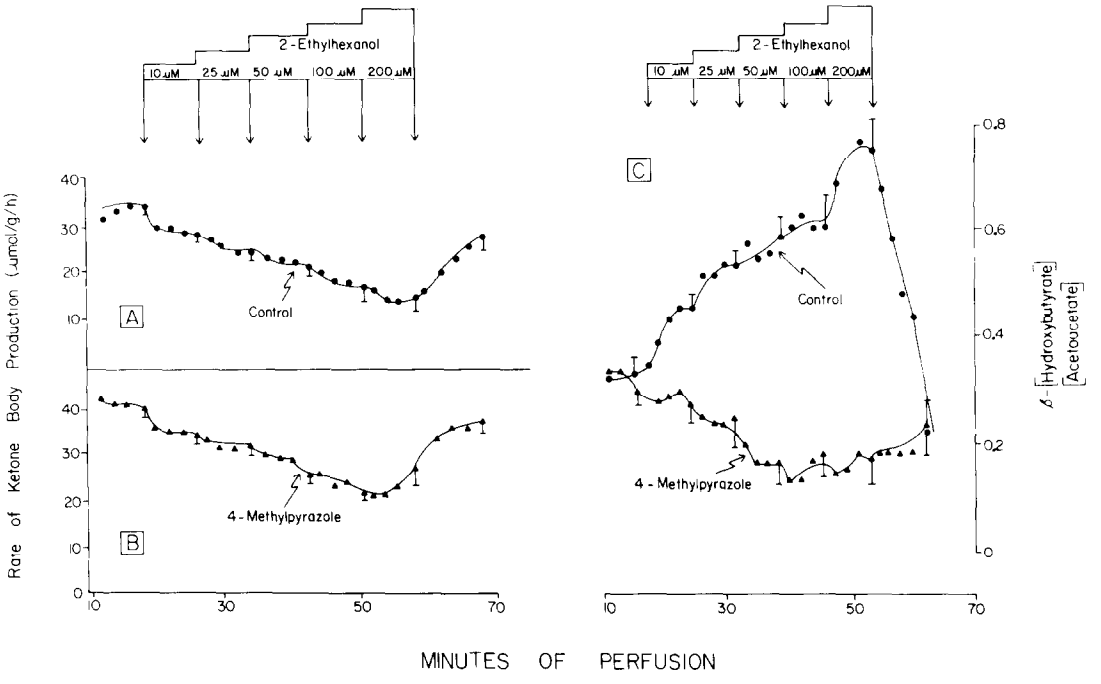

Fig. 1. Effect of ethylhexanol on ketone body production and mitochondrial NADH redox state. Livers from normal, starved rats were perfused in a nonrecirculating system as described in Materials and Methods. Rates of ketone body formation were calculated from effluent concentrations of acetoacetate + β-hydroxybutyrate, the flow rate, and the liver wet weight. Ethylhexanol was infused as indicated by the horizontal bars and vertical arrows. Key: (A) control; (B) experiment in the presence of 4-methylpyrazole (80 μM); and (C) β-hydroxybutyrate to acetoacetate ratios calculated from data depicted in A and B. Data are means ± SE for four livers per group.

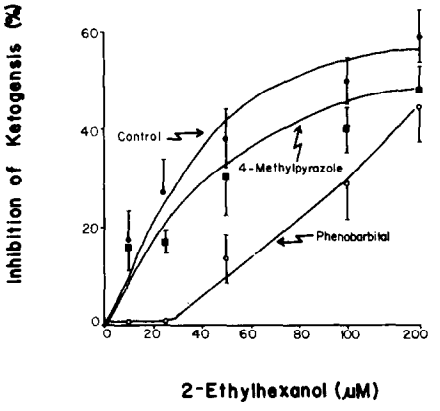

Fig. 2. Effect of 4-methylpyrazole and pretreatment with phenobarbital on ethylhexanol-induced inhibition of ketone body production by perfused rat liver. Rats were treated with phenobarbital (1 g/L) in drinking water for 7 days before use. Livers from starved rats were perfused in experiments as described in Fig. 1. Control and 4-methylpyrazole data are from Fig. 1. Data are means ± SE for four livers per group.

Effect of ethylhexanol on oxidation of long- and short-chain fatty acids. In the absence of added fatty acids, ethylhexanol inhibited ketogenesis by about 50% (Table 1). Infusion of the long-chain fatty acid oleate (250 μM) into livers from normal fasted rats increased rates of ketone body production from 39 ± 2 to 80 ± 6 μmol/g/hr (Table 1). Subsequent infusion of ethylhexanol diminished rates of ketogenesis significantly to 58 ± 8 μmol/g/hr. Hexanoate (250 μM), a short-chain fatty acid, increased rates of ketone body production to 95 ± 5 μmol/g/hr. In contrast to data obtained with oleate, these rates were not diminished significantly by ethylhexanol (82 ± 8; Table 1).

Effect of ethylhexanol on plasma ketone bodies and hepatic lipid content in vivo. Plasma ketone body concentrations were decreased significantly from 1.6 to 0.8 mM 100 min after intragastric administration of ethylhexanol (0.32 g/kg; Table 2). Osmium-positive material (lipid) was nearly undetectable in livers from control rats (Fig. 4A); however, increases in lipid content detected histologically were observed as early as 2 hr following administration of ethylhexanol (Fig. 4B). Lipid was in the form of microvesicular droplets which continued to accumulate in periportal regions of the liver lobule for at least 6 hr (Fig. 4C). Ethylhexanol increased both total hepatic lipid and triglyceride content *in vivo* significantly by 1.5- to 2-fold (Table 2).

DISCUSSION

Inhibition of β-oxidation by ethylhexanol. Ethylhexanol, a primary metabolite of the widely used plasticizer diethylhexyl phthalate, inhibited

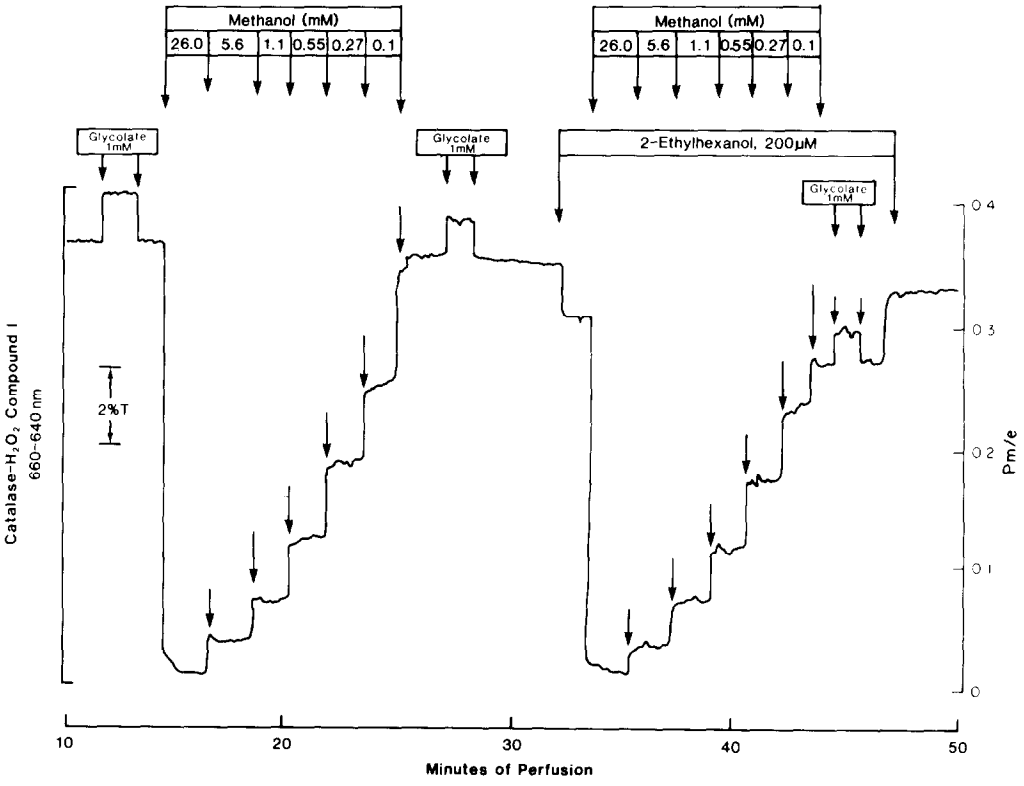

Fig. 3. Effect of methanol on the steady-state level of catalase-H_2O_2 in the presence and absence of ethylhexanol. The steady-state level of catalase-H_2O_2 was monitored spectrophotometrically (660 − 640 nm) as described in Materials and Methods. Glycolate (1 mM), methanol and ethylhexanol (200 μM) were infused as depicted by the horizontal bars and vertical arrows. Rates of H_2O_2 generation were calculated according to the method of Oshino *et al.* [19]. A typical experiment is presented.

Table 1. Inhibition of ketone body production from endogenous and exogenous fatty acids by 2-ethylhexanol in perfused rat liver

	Rates of Ketone body production (μmol/g/hr)	
Additions	Control	2-Ethylhexanol
None	39 ± 2	17 ± 3*
Oleate	80 ± 6†	58 ± 8‡
Hexanoate	95 ± 5†	82 ± 8

2-Ethylhexanol (200 μM) was infused into livers from starved rats, and ketone bodies were measured as described in Materials and Methods. Oleate or hexanoate (250 μM) was infused for 8 min followed by an infusion of 2-ethylhexanol simultaneously with fatty acid for an additional 8 min. Data are means ± SE for four livers per group.

* $P < 0.001$ compared with corresponding control value.

† $P < 0.001$ compared with basal value.

‡ $P < 0.05$ compared with corresponding control values.

hepatic ketone body production rapidly in a dose-dependent manner (Fig. 1). Since 2-ethylhexanol undergoes ω and ω-1 oxidation as well as alcohol dehydrogenase-mediated oxidation [22], we evaluated whether ethylhexanol *per se* or its metabolites were responsible for inhibition of ketogenesis. Ethylhexanol inhibited ketone body production to a similar extent in the presence and absence of 4-methylpyrazole, an inhibitor of alcohol dehydrogenase (Fig. 1 A and B); thus, metabolism of ethylhexanol by alcohol dehydrogenase is not obligatory for its inhibition of ketogenesis. Further, the mitochondrial NADH/NAD^+ redox state as reflected by the B/A ratio was not increased during inhibition of ketogenesis by ethylhexanol (Fig. 1); therefore, redox inhibition of fatty acid oxidation by elevated levels of NADH resulting from oxidation of ethylhexanol via alcohol dehydrogenase is unlikely.

Pretreatment of rats with phenobarbital induces cytochrome P450-dependent ω and ω-1 oxidation of the ethylhexanol moiety of diethylhexyl phthalate as well as the structurally similar branched chain fatty acid, valproate [23, 24]. Since the inhibitory effects of low doses of ethylhexanol (<50 μM) on ketogenesis were abolished by pretreatment of rats with phenobarbital (Fig. 2), it is likely that ethylhexanol is converted via ω and ω-1 oxidation to metabolites which do not inhibit ketogenesis. Taken together, the results indicate that the parent compound, ethylhexanol, and not a metabolite is responsible for the inhibition of ketone body production observed in this study.

Interaction between ethylhexanol and lipid metabolism in vivo. Addition of diethylhexyl phthalate to

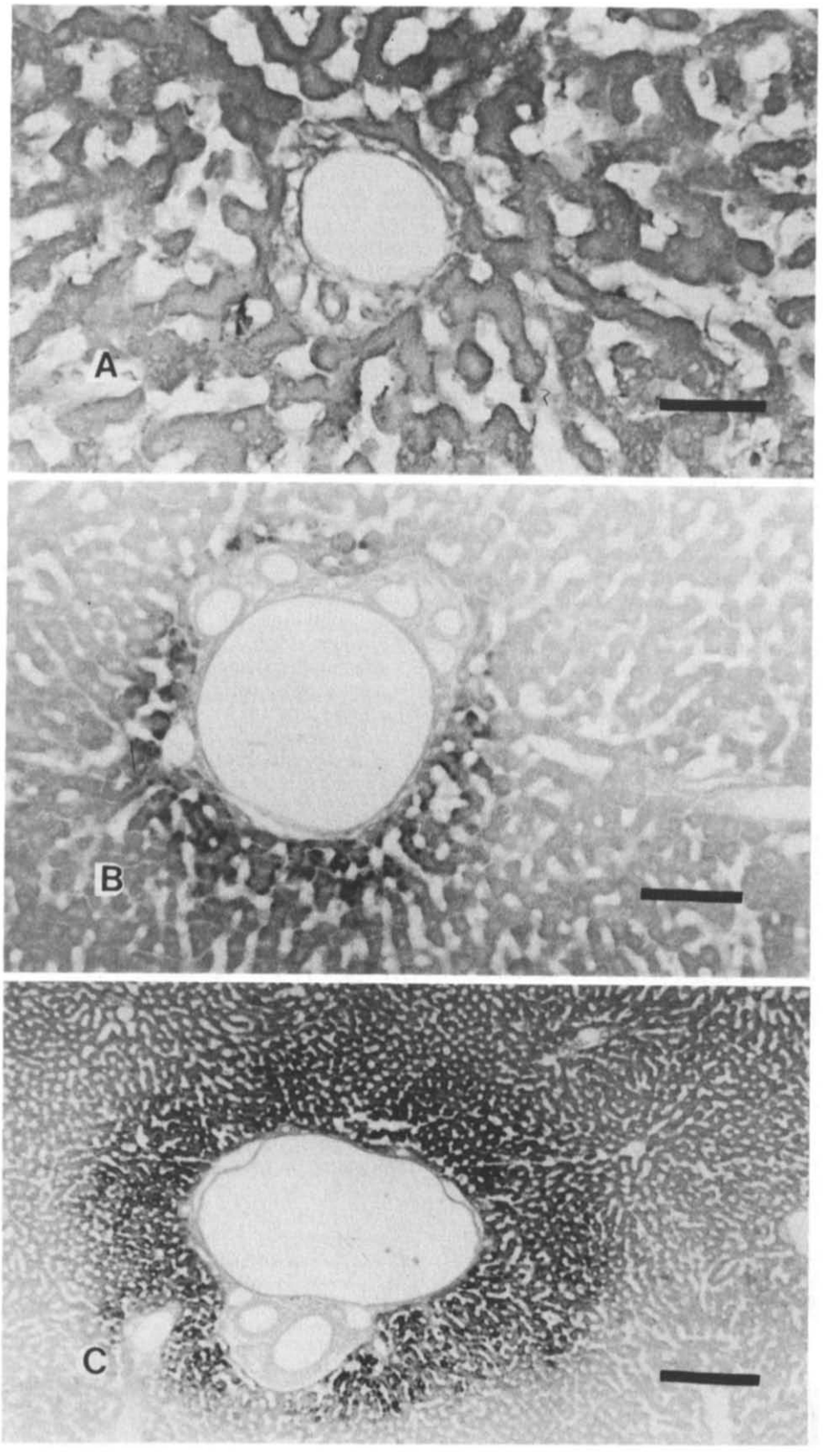

Fig. 4. Accumulation of lipid in livers from rats treated with ethylhexanol. Starved rats were treated with 2-ethylhexanol (0.1 g/kg, i.g.) for 0, 2, or 6 hr. Rats were then anesthetized with pentobarbital (50 mg/kg, i.p.) and livers were perfused briefly to remove blood and were fixed with 1% paraformaldehyde in Krebs–Henseleit buffer. Sections of tissue were stained subsequently with OsO_4 and eosin. Key: (A) liver from a rat given a corn oil vehicle (bar = 50 μm); (B) liver from a rat which received 2-ethylhexanol in corn oil for 2 hr (bar = 100 μm); and (C) liver from a rat given 2-ethylhexanol for 6 hr (bar = 250 μm).

Table 2. Effect of ethylhexanol on plasma ketone bodies and hepatic lipid *in vivo*

Treatment	Plasma ketones (mM)	Total lipids (mg/g)	Triglycerides (μmol/g)
Control	1.59 ± 0.07	27 ± 2	4.7 ± 0.2
Ethylhexanol	0.81 ± 0.6*	37 ± 3†	7.8 ± 1.1‡

Normal starved (24 hr) rats were given ethylhexanol (320 mg/kg) intraperitoneally. At 100 min, rats were killed and blood samples were collected. Plasma was analyzed for acetoacetate and β-hydroxybutyrate (ketone bodies) by standard enzymatic methods (see Materials and Methods). Values are means ± SE for six rats in each group. In a parallel group of rats, livers were freeze-clamped at 100 min, and lipids were determined gravimetrically in $CHCl_3$:CH_3OH extracts as described elsewhere. Triglycerides were determined enzymatically [21] by fluorometric determination of glycerol after hydrolysis with a lipase–esterase mixture.

*–‡ P values compared to appropriate control: * $P < 0.001$, † $P < 0.01$, and ‡ $P < 0.05$.

the diet increases the growth-promoting effects of fat and increases total lipid content in rat liver [24], suggesting an interaction between phthalates and lipid metabolism. Data presented above demonstrate that ethylhexanol decreased circulating levels of ketone bodies rapidly and increased hepatic lipid and triglyceride content *in vivo* (Fig. 4, Table 2). Lipid accumulated in the form of microvesicular droplets in periportal regions of the liver lobule, similar to that documented following treatment with peroxisome proliferating agents such as clofibrate and diethylhexyl phthalate [25]. These results are consistent with the hypothesis that inhibition of transport of acyl-CoA compounds into the mitochondria and/or diminished metabolism of triglyceride compounds leads to their accumulation predominantly in periportal regions of the liver lobule (Fig. 4).

Mechanism of inhibition of hepatic ketone body formation and accumulation of lipid due to ethylhexanol. Fatty acids are metabolized in liver by two oxidizing systems, one located in mitochondria and one in peroxisomes [6]. β-Oxidation of acyl-CoA compounds in peroxisomes generates one mole of H_2O_2 for each mole of acetyl-CoA produced, while mitochondrial β-oxidation produces NADH but not H_2O_2 [6]. Rates of H_2O_2 generation from either endogenous or added fatty acids were unaffected by ethylhexanol (Results), indicating that peroxisomal β-oxidation was not altered by ethylhexanol. Thus, inhibition of ketone body production by ethylhexanol is due to inhibition of mitochondrial β-oxidation of fatty acids. This inhibition may occur at several sites. First, ethylhexanol may inhibit activation of fatty acids to acyl-CoA compounds in the cytosol. This possibility seems unlikely, however, since lipid accumulated in the liver *in vivo* following administration of ethylhexanol (Table 2). Second, ethylhexanol may inhibit one or more enzymes of the mitochondrial β-oxidation system. This possibility also seems unlikely because ketone body formation from hexanoate, a fatty acid activated and metabolized predominantly in the mitochondria [26], was not inhibited by ethylhexanol. Third, ethylhexanol may decrease the transport of long-chain acyl-CoA compounds into mitochondria. This hypothesis may be explained by decreases in carnitine levels, by inhibition of carnitine acyl transferases or by increases in malonyl-CoA levels [27, 28]. For example, Foxworthy and Eacho [27] reported recently that the peroxisome proliferating agent 2-hydroxy-3-propyl-4-[6-(tetrazol-5-yl)hexyloxy]-acetophenone decreases mitochondrial β-oxidation by inhibition of carnitine acetyltransferase I. Further, valproic acid, a branched-chain fatty acid structurally similar to ethylhexanol, forms conjugates with carnitine [28]. The hypothesis that ethylhexanol inhibits the transfer of long-chain acyl-CoA compounds into mitochondria is further supported by the observation that ethylhexanol inhibited ketogenesis from endogenous, long-chain fatty acids as well as added oleate (Fig. 1, Table 1). Accumulation of total lipids and triglycerides after treatment with ethylhexanol further supports this hypothesis. Moreover, inhibition of ketogenesis and accumulation of hepatic lipid (Fig. 1, Table 2) precede increases in peroxisomal marker enzymes [29]. Elevation of lipid in the metabolically active periportal regions of the liver lobule may be involved in the mechanism of peroxisomal proliferation caused by plasticizers and their metabolites.

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