0026-895X/87/060631-07$02.00/0

MOLECULAR PHARMACOLOGY, 31:631-637

Diminished Pentose Cycle Flux in Perfused Livers of Ethanol-Fed Rats

LESTER A. REINKE, JOEL S. TUPPER, PHILLIP R. SMITH, and DAVID J. SWEENY

Department of Pharmacology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

Received November 24, 1986; Accepted March 3, 1987

SUMMARY

Rates of NADPH generation by the pentose phosphate pathway were evaluated in perfused livers from ethanol-fed or control rats by measuring the production of $^{14}CO_2$ from 1-^{14}C-glucose. Under basal perfusion conditions, livers from ethanol-fed rats released lactate and pyruvate into the perfusate at rates that were only 19% of the control values. Under these conditions, calculated rates of NADPH generation by the pentose cycle in livers of the ethanol-fed rats were only 50% of rates obtained with livers of control rats. 7-Ethoxycoumarin (7-EC), a substrate for mixed function oxidation, was infused to increase rates of hepatic NADPH utilization. In livers from control rats, 7-EC was oxidized at a rate of 2.6 μmol/g/hr, but rates of NADPH generation by the pentose cycle were increased by 8.8 μmol/g/hr. In livers from ethanol-fed rats, 7-EC was metabolized at rates of 7.2 μmol/g/hr, but the generation of NADPH by the pentose cycle was increased by only 3.9 μmol/g/hr. The infusion of 7-EC was associated with increases in rates of O_2 uptake that exceeded rates of mixed function oxidation in both groups of animals. Ethanol feeding decreased the activity of glucose-6-phosphate dehydrogenase by 40% and decreased the concentrations of glycogen by 66%. Thus, the decrease in pentose cycle flux in perfused livers may be due to diminished activity of the rate-controlling enzyme and/or diminished substrate supply from glycogen. However, cytosolic $NADP^+$/NADPH ratios were identical in livers of both groups. Because NADPH was not depleted during the mixed function oxidation of 7-EC in livers from ethanol-fed rats, it is concluded that other hepatic sources of NADPH compensate for the diminished generation by the pentose cycle.

Chronic abuse of ethyl alcohol often results in the development of liver toxicity. For this reason, the effects of acute and chronic ethanol administration on various aspects of hepatic metabolism have been studied extensively. Several excellent reviews are available which document the numerous effects that ethanol may exert on the metabolism of lipids (1), proteins (2), carbohydrates (3), and other aspects of intermediary metabolism (4–6). One area that has received less attention is a possible interaction between ethanol and the hepatic pentose phosphate cycle. Although the acute addition of ethanol to liver slices had little effect on pentose cycle activity (7), Harata *et al.* (8) have shown that chronic ethanol administration decreased the activity of hepatic glucose-6-phosphate dehydrogenase, the rate-limiting enzyme for this metabolic pathway. More recently, Mauch *et al.* (9) demonstrated that acetaldehyde inactivates glucose-6-phosphate dehydrogenase as a result of covalent binding at the catalytic site. In contrast, Luoma and Vorne (10) reported increased hepatic glucose-6-phosphate dehydrogenase activity after long-term administration of ethanol to rats in drinking water.

The pentose phosphate cycle is a major cytosolic source of NADPH, a cofactor required for mixed function oxidation, glutathione reductase, and various biosynthetic reactions. In addition, this pathway provides ribose required for the synthesis of various nucleotides. If ethanol feeding altered the activity of the pentose cycle, other pathways of hepatic metabolism could be affected by changes in cofactor supply. In order to evaluate this possibility, we have studied the effects of ethanol feeding on pentose cycle flux in perfused rat livers, both under basal conditions and during mixed function oxidation, which increases rates of NADPH utilization.

Materials and Methods

Animals. Female Sprague-Dawley rats (Sasco, Inc., Omaha, NE), weighing 160–180 g, were used in all experiments. Rats that were fed ethanol were given liquid diets containing ethanol and fat as 36% and 35% of total calories, respectively. The diets were prepared essentially as described in the 1982 formulations of Lieber and DeCarli (11), with the modification that AIN-76 (12) vitamin and mineral mixtures were utilized in the diet formulation. Pair-fed controls received liquid diets in which the ethanol calories were replaced by dextrin/maltose (11). All diet ingredients were purchased from Bioserve, Inc. (Frenchtown, NJ). The diets were administered at 4 p.m. daily for at least 2 weeks prior to experiments, and the animals were allowed free access to the diets until they were used in experiments.

Liver perfusion. Livers were removed from rats under pentobar-

This investigation was supported by United States Public Health Service Grant CA 30137, awarded by the National Cancer Institute, Department of Health and Human Services.

ABBREVIATIONS: 7-EC, 7-ethoxycoumarin; 7-HC, 7-hydroxycoumarin; GSSG, glutathione disulfide.

bital anesthesia and were perfused with Krebs-Henseleit bicarbonate buffer, pH 7.4, saturated with an oxygen/carbon dioxide mixture (95:5) in a nonrecirculating system (13). 7-EC (0.2 mM) was dissolved in the Krebs-Henseleit buffer prior to infusion. Glucuronide and sulfate conjugates of 7-HC were hydrolyzed by incubating 0.5 ml of perfusate with 2.0 ml of Tris buffer, pH 7.4, containing 250 units of purified β-glucuronidase and 25 units of sulfatase activity (Sigma). 7-HC was measured fluorimetrically, utilizing excitation and emission wavelengths of 380 nm and 490 nm, respectively (14).

Measurement of pentose phosphate cycle activity. When glucose is metabolized by the pentose cycle, the carbon at position 1 is lost as CO_2. For this reason, the formation of $^{14}CO_2$ from 1-^{14}C-glucose can be utilized to estimate rates of pentose cycle flux. However, metabolism of 1-^{14}C-glucose by glycolysis and the citric acid cycle also produces $^{14}CO_2$. Two experimental designs have been utilized to differentiate $^{14}CO_2$ produced from the pentose cycle in these studies. In the first design, 1-^{14}C-glucose and 6-^{14}C-glucose were separately infused into livers under identical experimental conditions. The 6-^{14}C-glucose was used to estimate $^{14}CO_2$ formed from non-pentose cycle pathways, and the difference in $^{14}CO_2$ production from the radiotracers was assumed to arise from the pentose cycle.

The alternate experimental design was taken from the method of Kuehn and Scholz (15), and is illustrated in Fig. 1. 1-^{14}C-Glucose was infused for 8 min, and samples of perfusate were taken at 1-min intervals for $^{14}CO_2$ measurements. After ^{14}C-glucose infusion was terminated, additional samples were taken to determine the washout kinetics of $^{14}CO_2$. Under these conditions, two distinct kinetic components, designated as I and II (Fig. 1), could be detected. Component I has been identified as a rapid decrease in $^{14}CO_2$ arising from the pentose cycle, while $^{14}CO_2$ represented by component II originates in the citric acid cycle (15). By extrapolating component II back to the time that 1-^{14}C-glucose infusion was terminated, the $^{14}CO_2$ arising from the pentose cycle (Fig. 1, *PC*) under steady state conditions can be estimated. This technique has been shown to give similar estimates of pentose cycle-dependent $^{14}CO_2$ production as the more traditional approach of comparing data obtained with 1-^{14}C- and 6-^{14}C-glucose (15). This kinetic method also facilitates measurements of pentose cycle activity in the absence and presence of 7-EC in the same liver (Fig. 1). Rates of pentose cycle flux can be determined from $^{14}CO_2$ arising from the pentose cycle, the specific activity of lactate in perfusate, and rates of lactate plus pyruvate efflux from the perfused liver (15). Rates of NADPH production were calculated from the assumption that 2 mol of NADPH were produced per mol of CO_2 formed by the pentose cycle.

In all pentose cycle experiments, the ^{14}C-glucose was purified prior to use by anion exchange chromatography (15) and was infused into livers at a rate of 1.1 μCi/min. Glucose (10 mM) was added to the Krebs-Henseleit bicarbonate buffer in pentose cycle experiments to serve as a carrier for the radiotracer. Samples of perfusate (20 ml) were added to stoppered 50-ml flasks containing 1 ml of 2 N HCl. $^{14}CO_2$ released was trapped in phenethylamine which was suspended above the perfusate samples in disposable center wells (Kontes). Lactate was isolated from perfusate fractions by anion exchange chromatography. In initial experiments, the lactate was eluted from Dowex columns with 0.6 N formic acid, as described previously (15). However, it was found subsequently that fractions containing lactate were contaminated with another unidentified radioactive metabolite. Elution of lactate with 0.1 N formic acid resolved lactate from the contaminating substance.

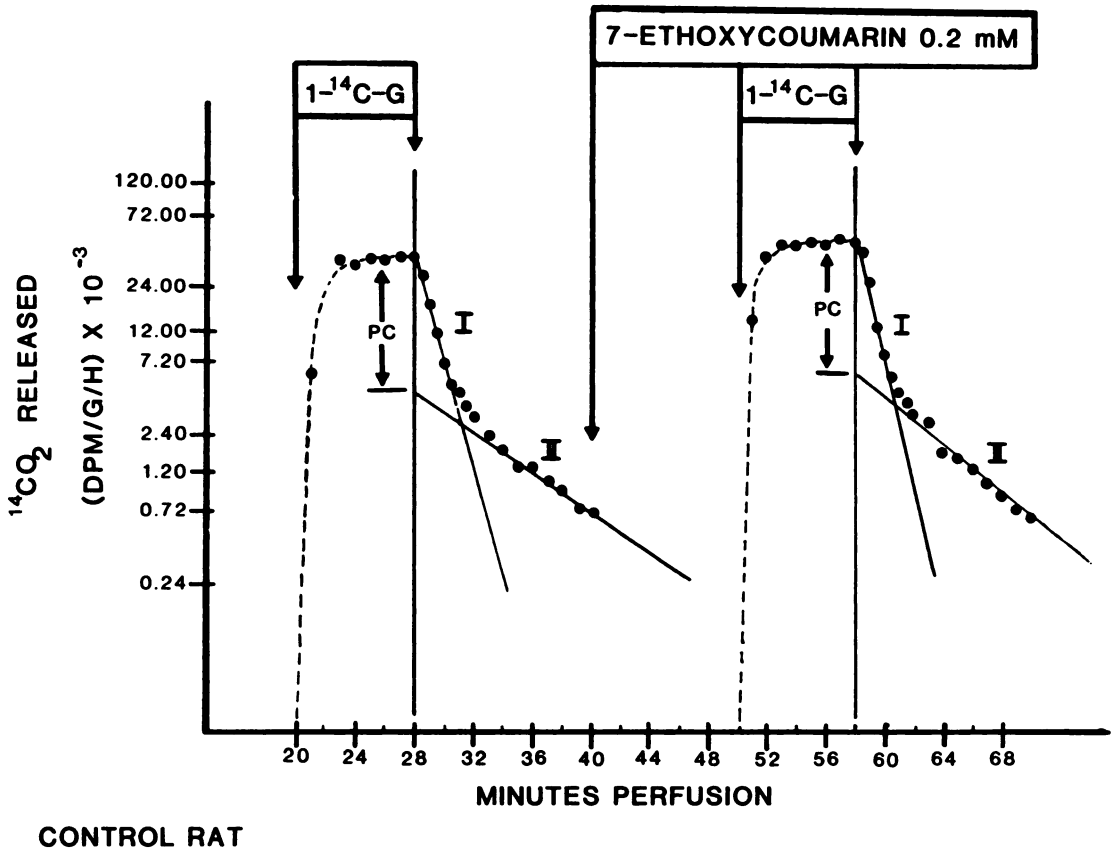

Fig. 1. $^{14}CO_2$ formation from 1-^{14}C-glucose in the absence and presence of 7-EC. Livers were preperfused with Krebs-Henseleit bicarbonate buffer, which also contained 10 mM glucose, for 20 min before 1-^{14}C-glucose (*1-^{14}C-G*, 1.1 μCi/min) was infused. Intervals of ^{14}C-glucose and 7-EC infusions are indicated by the *horizontal bars* and *vertical arrows*. Samples of perfusate were taken for $^{14}CO_2$ determinations at the times indicated (●). The significance of kinetic components I and II is discussed under Materials and Methods. *PC* indicates the $^{14}CO_2$ calculated to arise from the pentose cycle under steady state conditions. These data are typical for experiments conducted with livers of rats fed the liquid control diet.

Radioactivity was quantitated by liquid scintillation spectrophotometry, utilizing a Packard Tri-Carb 460 CD Scintillation System.

Measurement of enzyme activities and metabolic intermediates. Glucose, lactate, and pyruvate in the perfusate were measured enzymatically (16). In some experiments, perfused livers were freeze-clamped with tongs chilled in liquid nitrogen, and pentose cycle intermediates were measured in $HClO_4$ extracts by standard enzymatic methods (16, 17). NADPH concentrations in alkaline liver extracts were determined by the method of Giblin and Reddy (18). GSSG was measured according to the method of Akerboom and Sies (19). Glycogen was isolated according to the method of Hassid and Abraham (20); the glucosyl units were hydrolyzed in 1 N HCl, and glucose was measured enzymatically. Hepatic glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity was measured as described by Löhr and Waller (see Ref. 16), isocitrate dehydrogenase (EC 1.1.1.42) activity was assayed by the method of Bernt and Bergmeyer (see Ref. 16), and 6-phosphogluconate dehydrogenase (EC 1.1.1.43) and "malic" enzyme (EC 1.1.1.38) activities were measured as described by Luine and Kauffman (21).

Results

Rates of mixed function oxidation of 7-EC in perfused livers from ethanol-fed and control rats. When 7-EC (0.2 mM) was infused into a liver from an ethanol-fed rat, 7-HC was released into perfusate as the free metabolite, or as glucuronide and sulfate conjugates (Fig. 2A). After 16–18 min of continued 7-EC infusion, 7-HC was formed at a steady state rate of approximately 8 μmol/g/hr. In a perfused liver from a rat fed the liquid control diet, the rate of 7-HC formation reached only 3 μmol/g/hr (Fig. 2B). The higher rate of 7-EC metabolism in livers of ethanol-fed rats was anticipated because hepatic 7-EC *O*-deethylase activity has been shown to be highly inducible by ethanol feeding (22). The infusion of 7-EC also caused increases in hepatic O_2 uptake of 20–25 μmol/g/hr in the liver of the ethanol-fed rat, and 10–15 μmol/g/hr in the liver of the control (Fig. 2).

Evaluation of pentose phosphate cycle activity in perfused livers from ethanol-fed and control rats. 1-^{14}C-glucose and 6-^{14}C-glucose were separately infused into livers from ethanol-fed and control rats, and rates of $^{14}CO_2$ formation were plotted on the same time scale for easy comparison (Fig. 3). In the absence of 7-EC, rates of $^{14}CO_2$ formation from 1-^{14}C-glucose were similar in livers from ethanol-fed and control rats (38,000–40,000 dpm/g/hr). When 7-EC was added, the rates of $^{14}CO_2$ formation from 1-^{14}C-glucose increased by about 75% in the liver from the control rat but increased only about 30% in the liver of the ethanol-fed rat (Fig. 3).

The difference in $^{14}CO_2$ formation from 1-^{14}C-glucose and 6-^{14}C-glucose can be used to provide an estimation of pentose cycle activity. Using this approach, the data in Fig. 3A suggest that the pentose cycle produced $^{14}CO_2$ from 1-^{14}C-glucose at a rate of about 33,700 dpm/g/hr under basal conditions and 39,700 dpm/g/hr in the presence of 7-EC. The comparable values obtained from the control liver were 35,300 dpm of $^{14}CO_2$/g/hr under basal conditions and 55,400 dpm/g/hr in the presence of 7-EC. Thus, these data provide preliminary evidence that pentose cycle flux in livers of ethanol-fed rats may be diminished by about 30% from control values during the metabolism of 7-EC. In these experiments, rates of 7-EC metabolism were 7.1 and 1.6 μmol/g/hr in livers of the ethanol-fed and the control rat, respectively.

In subsequent experiments, rates of pentose cycle flux were

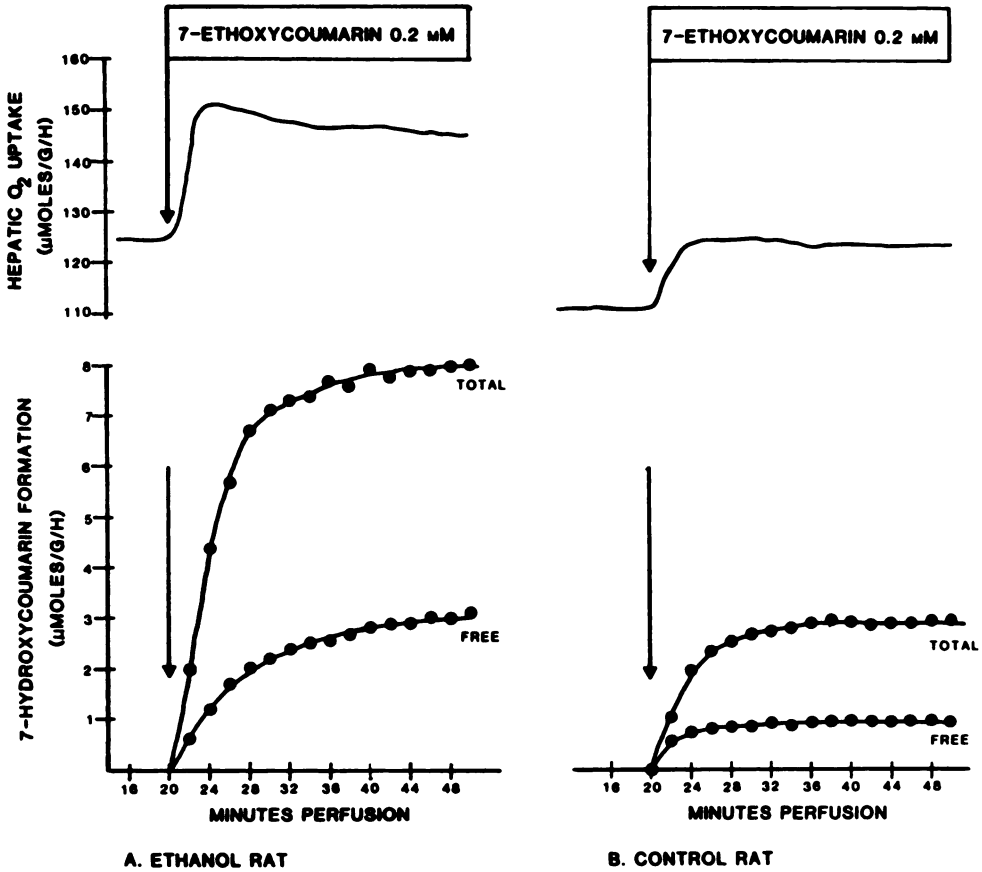

Fig. 2. Rates of 7-HC formation and hepatic respiration during infusion of 7-EC into perfused rat livers. 7-EC (0.2 mM) was infused after 20 min of preperfusion with Krebs-Henseleit bicarbonate buffer. The oxygen concentration in the venous effluent was monitored continually with a Clark-type oxygen electrode. Concentrations of 7-HC in perfusate were measured fluorimetrically both after enzymatic hydrolysis of glucuronide and sulfate conjugates (*TOTAL*) and without enzymatic hydrolysis (*FREE*). Rates of O_2 uptake and 7-HC formation were calculated from concentrations in perfusate, the flow rate, and the liver wet weight. A. Liver from an ethanol-fed rat. B. Liver from a rat fed the liquid control diet.

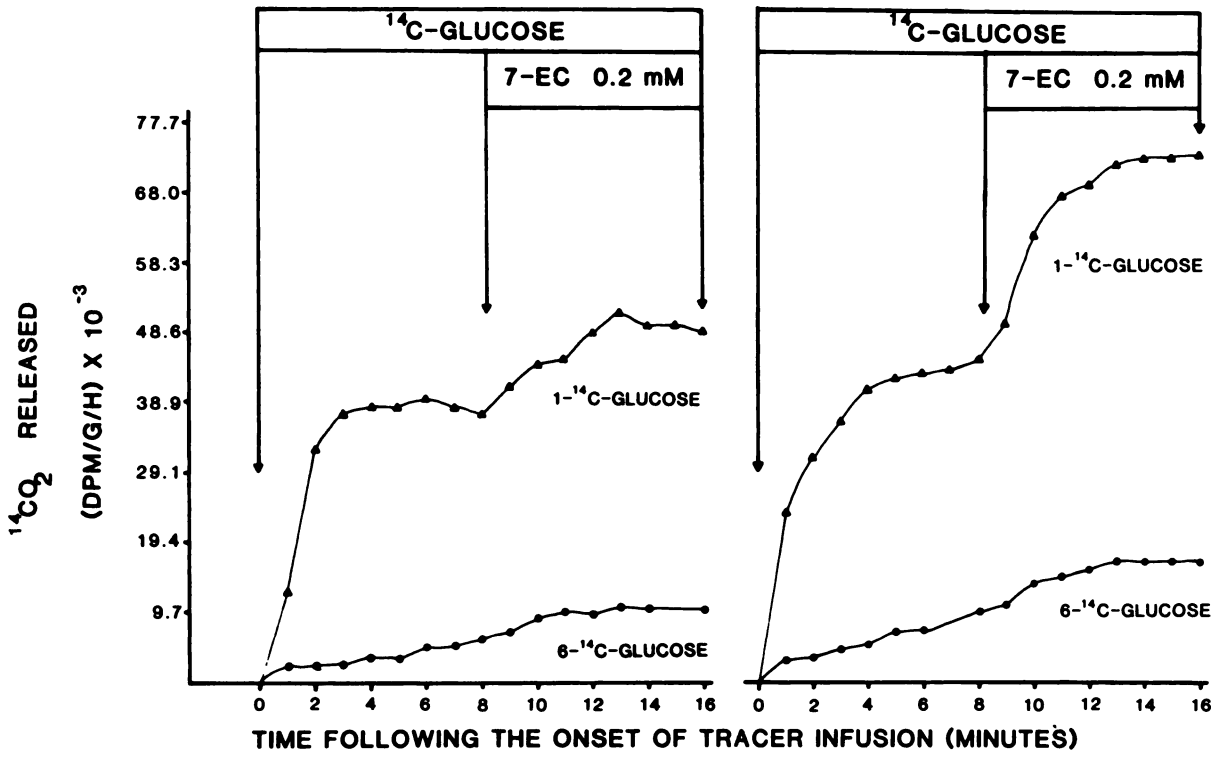

Fig. 3. Effect of 7-EC infusion on rates of $^{14}CO_2$ formation from 1-^{14}C-glucose and 6-^{14}C-glucose in perfused rat livers. Both radiotracers were purified on Dowex-formate columns (15) and were infused into livers at a rate of 1.1 μCi/min. Samples of perfusate were collected for $^{14}CO_2$ measurements during 8 min of 1-^{14}C-glucose infusion, and then 7-EC (0.2 mM) was infused. After 8 min, the infusions of both 7-EC and 1-^{14}C-glucose were stopped and perfusion was continued with plain buffer to allow $^{14}CO_2$ and 7-EC to diffuse from the liver. After 14 min, this sequence was repeated with 6-^{14}C-glucose. Data obtained with both radiotracers were plotted on the same time scale for easy comparison. ▲, $^{14}CO_2$ formed from 1-^{14}C-glucose; ●, $^{14}CO_2$ from the subsequent infusion of 6-^{14}C-glucose. A. Ethanol-fed rat. B. Rat fed liquid control diet.

measured by the washout kinetic method of Kuehn and Scholz (15), which is illustrated in Fig. 1. In the absence of 7-EC, the rate of $^{14}CO_2$ formation by the pentose cycle in livers from ethanol-fed rats (33,960 dpm/g/hr) was only slightly less than the corresponding control values (40,143 dpm/g/hr, Table 1). Under these conditions, rates of lactate plus pyruvate release from livers of ethanol-fed rats were only 19% of the control values. The specific activity of lactate isolated from perfusate was 30% higher in the case of the ethanol-fed rats (Table 1). From these data, calculated rates of NADPH generation from the pentose cycle under basal perfusion conditions were 22.3 μmol/g/hr in livers of control rats, but only 12.3 μmol/g/hr in livers of the ethanol-fed rats (Table 1).

The addition of 7-EC increased rates of lactate plus pyruvate formation, pentose cycle $^{14}CO_2$ production, and NADPH generation in both groups of rats, but the increases were greater in the livers of the controls (Table 1). Rates of lactate plus pyruvate formation in livers of ethanol-fed rats remained only 28% of control values, and rates of pentose cycle NADPH generation were about half the control values in the presence of 7-EC (Table 1). Under these conditions, the average rate of 7-HC formation was increased nearly 3-fold by chronic ethanol feeding. The addition of 7-EC increased rates of hepatic O_2 uptake (Fig. 1) by 29.9 μmol/g/hr in livers of ethanol-fed rats, but only by 17.4 μmol/g/hr in livers of controls (Table 1).

Activity of NADPH-generating enzymes and concentrations of pentose cycle intermediates in livers from ethanol-fed and control rats. Chronic ethanol feeding decreased the activity of hepatic glucose-6-phosphate dehydrogenase to 59% of the control values (Table 2), but 6-phosphogluconate dehydrogenase activity was unchanged. Isocitrate dehydrogenase had the highest activity of all hepatic NADPH-generating enzymes (Table 2) and its activity was also diminished by 22% in livers from ethanol-fed rats. "Malic" enzyme had the lowest activity of the enzymes measured and was not affected by ethanol feeding.

Various intermediates related to pentose cycle activity were measured in freeze-clamped livers from ethanol-fed and control rats, which had been perfused with 7-EC. Hepatic glycogen levels were markedly decreased in perfused livers from ethanol-fed rats (12.1 versus 35.8 μmol/g, Table 3). Concentrations of glucose 6-phosphate in livers of ethanol-fed rats were only 43% of control values, but levels of 6-phosphogluconate and ribulose-5-phosphate were unchanged. $NADP^+$/NADPH ratios calculated from the equilibrium constant for 6-phosphogluconate dehydrogenase (23) were also the same in the two groups (Table 3). In related experiments, similar concentrations of NADPH were measured in alkaline extracts of livers from three ethanol-fed rats (140.8 ± 9.3 nmol/g) and three control rats (119.5 ± 20.1 nmol/g).

Discussion

Diminished pentose phosphate cycle activity in perfused livers from ethanol-fed rats. Two important differ-

TABLE 1

Rates of lactate and pyruvate efflux and generation of NADPH by the pentose cycle in perfused livers from ethanol-fed and control rats

The experimental design is shown in Fig. 1. After an initial infusion of 1-^{14}C-glucose for pentose cycle measurements, 7-EC was infused for 10 min prior to a second infusion of 1-^{14}C-glucose. The infusion of 7-EC was associated with an increase in hepatic O_2 uptake (Fig. 2). Rates of 7-HC formation are the sum of the free and conjugated metabolites (Fig. 2). Concentrations of lactate and pyruvate formed during 1-^{14}C-glucose infusion were measured enzymatically, and rates of production were calculated from the perfusate concentration, the flow rate, and liver wet weight. Techniques utilized for the determination of $^{14}CO_2$ from the pentose cycle and rates of NADPH generation are described in detail under Materials and Methods. Values are means ± SE from 12 experiments, except that values for specific activity of lactate and NADPH generation are based on six experiments in which 0.1 N formic acid elution was used to separate radiolabeled lactate from an unidentified radioactive metabolite during anion exchange chromatography (see Materials and Methods).

Parameter measured	7-EC	Ethanol-fed rats	Control rats
Lactate plus pyruvate (μmol/g/hr)	−	18.1 ± 2.4[a]	95.7 ± 7.1
	+	42.6 ± 6.0[a]	149.3 ± 13.6
$^{14}CO_2$ from the pentose cycle (dpm/g/hr)	−	33,960 ± 2,501	40,143 ± 2,002
	+	35,081 ± 5,156[b]	52,224 ± 3,280
Specific activity of lactate (dpm/μmol)	−	2,178 ± 242	1,706 ± 191
	+	2,031 ± 184[b]	1,451 ± 84
NADPH generation by pentose cycle (μmol/g/hr)	−	12.3 ± 1.3[a]	22.3 ± 2.4
	+	16.2 ± 2.6[a]	31.1 ± 3.2
Rate of 7-HC formation (μmol/g/hr)	+	7.2 ± 0.8[a]	2.6 ± 0.3
Increase in oxygen uptake during 7-EC infusion (μmol/g/hr)	+	29.9 ± 4.3[b]	17.4 ± 3.7

[a] $p < 0.005$ with respect to control values.
[b] $p < 0.05$ with respect to control values.

TABLE 2

Activities of NADPH-generating enzymes in liver homogenates from ethanol-fed or control rats

Values are nmol of NADPH formed/min/mg of protein from five rats per group. Enzyme activities were measured as described in Materials and Methods.

Enzyme activity	Ethanol-fed rats	Control rats
Glucose-6-phosphate dehydrogenase	21.3 ± 3.0[a]	36.0 ± 1.5
6-Phosphogluconate dehydrogenase	41.3 ± 2.0	45.5 ± 4.7
Isocitrate dehydrogenase	216.6 ± 13.4[b]	276.2 ± 21.4
"Malic" enzyme	8.7 ± 0.4	16.9 ± 3.1

[a] $p < 0.005$ with respect to control values.
[b] $p < 0.05$ with respect to control values.

ences in 1-^{14}C-glucose metabolism were observed in experiments with perfused livers from ethanol-fed or control rats. First, rates of $^{14}CO_2$ formation from the pentose cycle were greater in livers from control rats, especially during the mixed function oxidation of 7-EC. This same conclusion could be reached if experiments were conducted by washout kinetic methods (Table 1), or by comparing $^{14}CO_2$ produced during infusion of 1-^{14}C- and 6-^{14}C-glucose (Fig. 3). A second observation is that the average specific activity of lactate isolated from perfusate was always higher in the case of the ethanol-fed rats (Table 1).

In order to calculate rates of pentose cycle activity from $^{14}CO_2$ production, the specific activity of the precursor from which the $^{14}CO_2$ is produced must be known. One approach for these calculations is to assume that the specific activity of intracellular 6-phosphogluconate is equal to the specific activity of the glucose infused (approximately 5800 dpm/μmol in these experiments). For example, if pentose cycle activity in the

TABLE 3

Hepatic concentrations of glycogen, pentose cycle intermediates, and glutathione disulfide in perfused livers from ethanol-fed and control rats

Livers were preperfused for 20 min with Krebs-Henseleit bicarbonate buffer, containing 10 mM glucose, and then 7-EC (0.2 mM) was infused. After 10 min of 7-EC infusion, the livers were freeze-clamped with tongs chilled in liquid N_2 and stored at −80°. The intermediates were measured as described in Materials and Methods. The free cytosolic NADP$^+$/NADPH ratios were calculated from the measured concentrations of 6-phosphogluconate and ribulose-5-phosphate, assuming an equilibrium constant of 0.17 M for 6-phosphogluconate and a CO_2 concentration of 1.16 mM (23). Values are means ± SE from five rats per group.

Parameter measured	Ethanol-fed rats	Control rats
Glycogen (μmol/g)	12.1 ± 7.1[a]	35.8 ± 6.9
Glucose 6-phosphate (nmol/g)	54.8 ± 8.1[a]	126.0 ± 41.7
6-Phosphogluconate (nmol/g)	4.0 ± 0.6	4.8 ± 1.4
Ribulose-5-phosphate (nmol/g)	9.6 ± 1.2	9.2 ± 1.9
Cytosolic NADP$^+$/NADPH	0.019 ± 0.004	0.016 ± 0.005
Glutathione disulfide (nmol/g)	234.0 ± 16.9	266.3 ± 14.4

[a] $p < 0.05$ with respect to controls.

presence of 7-EC were calculated from the $^{14}CO_2$ data shown in Table 1 and the specific activity of glucose infused, rates of NADPH generation of 12.1 and 18.0 μmols/g/hr would be obtained for livers of ethanol-fed and control rats, respectively. However, this simplified approach does not take into account the dilution of influent ^{14}C-glucose by the breakdown of glycogen, or recycling of pentose cycle products via gluconeogenesis. Because livers from control rats have higher concentrations of glycogen and glucose 6-phosphate (Table 3), it follows that the ^{14}C-glucose is diluted to a greater extent in livers from controls. As a result, calculation of pentose cycle flux from the specific activity of influent glucose will underestimate pentose cycle activity, especially in livers from control animals.

The formation of ^{14}C-lactate during infusion of 1-^{14}C-glucose can only result from the glycolytic metabolism of glucose, since the ^{14}C is lost if metabolism occurs via the pentose pathway. Kuehn and Scholz (15) have developed a method to estimate the specific activity of intracellular glucose 6-phosphate, utilizing the specific activity of lactate isolated from the perfusate and rates of release of lactate, pyruvate, and $^{14}CO_2$. For example, the calculated average specific activity of glucose 6-phosphate in livers of ethanol-fed rats during 7-EC infusion was 4037 ± 487 dpm/μmol, while the corresponding control value was 3204 ± 166 dpm/μmol (data not shown). This approach provides a more realistic approximation of the specific activity of cellular 6-phosphogluconate than the specific activity of glucose infused. From these calculations, the data indicate that rates of NADPH generation by the pentose cycle in livers from ethanol-fed rats were decreased from control values by 50% under both basal perfusion conditions and under conditions of NADPH demand for the mixed function oxidation of 7-EC (Table 1).

Two mechanisms can be proposed for diminished pentose cycle flux in livers from ethanol-fed rats. First, the activity of glucose-6-phosphate dehydrogenase, the initial enzyme in the pentose cycle, was depressed by 40% in livers of ethanol-fed rats (Table 2). Harata *et al.* (8) have also reported a 35% decrease in the activity of this enzyme after 4 weeks of ethanol feeding. Second, the supply of pentose cycle substrate from glycogen was diminished in livers from ethanol-fed rats (Table

3). Fasting has been shown to decrease both hepatic glycogen stores and pentose cycle flux (15, 24). Decreases in hepatic glycogen concentrations following ethanol feeding have been attributed to increased glycogen phosphorylase activity (25). The observed decreases in hepatic concentrations of glucose 6-phosphate (Table 3) and efflux of lactate and pyruvate (Table 1) from livers of ethanol-fed rats are probably also related to diminished glycogen stores.

Eggleston and Krebs (26) have proposed that GSSG may have a regulatory role on glucose-6-phosphate dehydrogenase activity. However, hepatic concentrations of GSSG were identical in both groups of rats (Table 3) and, thus, cannot explain the observed differences in pentose cycle activity. Another possible explanation for impaired pentose cycle activity following ethanol feeding is inhibition of glucose-6-phosphate dehydrogenase activity by fatty acids (27) or fatty acyl-CoA intermediates (28). Because administration of ethanol in a high fat diet leads to development of fatty liver (11), interactions between ethanol, dietary fat, and pentose cycle flux are being evaluated further.

Even though the data from these experiments indicated that rates of NADPH generation by the pentose cycle were depressed in livers from ethanol-fed rats, it must be emphasized that sufficient NADPH was available for mixed function oxidation. For example, high rates of 7-EC metabolism were sustained in perfused livers from ethanol-fed rats (Fig. 2) and $NADP^+$/NADPH ratios were identical in both groups of rats (Table 3). Mitochondria are known to be alternative sources for cytosolic NADPH, and their role in providing reducing equivalents for mixed function oxidation in perfused livers has been established (29). Because low rates of lactate and pyruvate release from perfused livers of ethanol-fed rats (Table 1) are indicative of diminished glucose catabolism, it is reasonable to assume that the mitochondrial oxidation of fatty acids may serve as an alternative energy source. This possibility is supported by the observation that rates of acetoacetate and β-hydroxybutyrate efflux were increased 3-fold over control values by ethanol feeding (data not shown). Both isocitrate dehydrogenase and "malic" enzyme derive their substrates from mitochondrial oxidations and could conceivably contribute to NADPH generation when pentose cycle activity is inadequate. Isocitrate dehydrogenase has the higher catalytic activity (Table 2) and has been proposed as a major source of NADPH required for mixed function oxidation (30).

Stoichiometries of mixed function oxidation, oxygen utilization, and pentose cycle-dependent NADPH generation in perfused rat livers. Mixed function oxidation is a process with a theoretical stoichiometry of NADPH/drug oxidized/O_2 of 1:1:1 (31). However, experiments with isolated microsomes and purified cytochrome P-450 isozymes have indicated that O_2 may also be enzymatically reduced to hydrogen peroxide and water, at the expense of NADPH (32, 33). Because the mixed function oxidation of 7-EC in perfused livers is associated with increased respiration and pentose cycle-dependent NADPH generation, it was of interest to compare these stoichiometries in the intact perfused organ.

In livers from control rats, 7-EC was metabolized at a rate of 2.6 μmol/g/hr, but NADPH generation was increased by 8.8 μmol/g/hr (Table 1). Thus, the increase in NADPH generation exceeded rates of NADPH utilization for mixed function oxidation by more than 3-fold. Under these conditions, the increase in O_2 uptake was 17.4 μmol/g/hr (Table 1). Although many explanations could be proposed for the excessive utilization of O_2, it is likely that at least some was converted into H_2O_2, as has been shown to occur during the metabolism of ethylmorphine in isolated hepatocytes (34). Cytosolic H_2O_2 is metabolized primarily by glutathione peroxidase (34), which results in the formation of water and GSSG. Additional NADPH would then be required to reduce GSSG by the glutathione reductase reaction. This sequence of events is consistent with data obtained in livers from control rats, in which the pentose cycle-dependent NADPH generation and O_2 utilization exceeded the requirements for 7-EC metabolism. A similar phenomenon has been reported to occur during the metabolism of *p*-nitroanisole in livers from phenobarbital-treated rats (24).

In livers of ethanol-fed rats, 7-EC infusion caused increases in NADPH generation by the pentose cycle (3.9 μmol/g/hr) which were insufficient to support the rate of 7-EC metabolism (7.2 μmol/g/hr, Table 1). Moreover, the increase in O_2 utilization during 7-EC metabolism (29.9 μmol/g/hr, Table 1) was even greater than the increase in O_2 uptake observed in control livers. If some of this O_2 is converted into H_2O_2 in livers of ethanol-fed rats, it can be questioned whether the H_2O_2 is adequately catabolized when the pentose cycle pathway is impaired. Indeed, oxidative damage has been proposed as a mechanism for ethanol-induced hepatotoxicity (35). On the other hand, the ethanol-inducible isozyme of cytochrome P-450 in rabbit liver has been shown to be the most effective isozyme studied in reducing O_2 directly to water (33). However, the reduction of O_2 to either H_2O_2 or water will increase the requirements for reducing equivalents, which apparently arise from sources other than the pentose cycle in livers from ethanol-fed rats.

In summary, experiments with perfused rat livers have indicated that ethanol feeding diminishes hepatic pentose cycle activity under basal conditions and during mixed function oxidation. Because the pentose pathway is a major source of NADPH and also provides ribose required for nucleotide synthesis, these observations may have important implications for the long-term effects of ethanol consumption on hepatic function.

References

1. Lieber, C. S., and M. Savolainen. Ethanol and lipids. *Alcoholism (Zagreb)* **8**:409–423 (1984).
2. Lieber, C. S. Alcohol, liver injury and protein metabolism. *Pharmacol. Biochem. Behav.* **13**(Suppl. 1):17–30 (1980).
3. Tejwoni, G. A., and V. A. Duruibe. Effect of ethanol on carbohydrate metabolism, in *Regulation of Carbohydrate Metabolism* (R. Beitner, ed.), Vol. II. CRC Press, Boca Raton, 67–93 (1985).
4. Reitz, R. C. Effects of ethanol on the intermediary metabolism of liver and brain, in *Biochemistry and Pharmacology of Alcohol* (E. Majchrowicz and E. P. Noble, eds.). Plenum Press, New York, 353–382 (1979).
5. Orrego, H., Y. Israel, and L. M. Blendis. Alcoholic liver disease: information in search of knowledge. *Hepatology (Baltimore)* **1**:267–283 (1981).
6. Lieber, C. S. Alcohol and the liver: 1984 update. *Hepatology (Baltimore)* **6**:1243–1260 (1984).
7. Majchrowicz, E., and J. H. Quastel. Effects of aliphatic alcohols on the metabolism of glucose and fructose in rat liver slices. *J. Biochem. Physiol.* **41**:793–803 (1963).
8. Harata, J., M. Nagata, E. Sasaki, I. Ishigura, Y. Ohta, and Y. Murakami. Effect of prolonged alcohol administration on activities of various enzymes scavenging activated oxygen radicals and lipid peroxide level in the liver of rats. *Biochem. Pharmacol.* **32**:1795–1798 (1983).
9. Mauch, T. J., T. M. Donohue, R. K. Zetterman, M. F. Sorrel, and D. J. Tuma. Covalent binding of acetaldehyde selectively inhibits the catalytic activity of lysine-dependent enzymes. *Hepatology (Baltimore)* **6**:263–269 (1986).
10. Luoma, P., and M. Vorne. Changes in the hepatic microsomal enzyme activity during long-term ethanol feeding in rat. *Acta Pharmacol. Toxicol.* **38**:260–266 (1976).

11. Lieber, C. S., and L. M. DeCarli. The feeding of alcohol in liquid diets: two decades of applications and 1982 update. *Alcoholism (Zagreb)* **6**:503–531 (1982).
12. Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. *J. Nutr.* **107**:1340–1348 (1977).
13. Scholz, R., W. Hansen, and R. G. Thurman. Interaction of mixed-function oxidation with biosynthetic processes. Inhibition of gluconeogenesis by aminopyrine in perfused rat liver. *Eur. J. Biochem.* **38**:64–72 (1973).
14. Al-Turk, W. A., and L. A. Reinke. Diminished conjugation of products of mixed-function oxidation in perfused livers from hypophysectomized rats. *Pharmacology* **27**:74–84 (1983).
15. Kuehn, A., and R. Scholz. Rates of flux through the pentose cycle in perfused rat liver: a procedure for the calculation of substrate flux from $^{14}CO_2$ production from 1-^{14}C-glucose. *Eur. J. Biochem.* **124**:611–617 (1982).
16. Bergmeyer, H. U. *Methods of Enzymatic Analysis*, 2nd English translation. Academic Press, New York (1974).
17. Lowry, O. H., and J. V. Passonneau. *A Flexible System of Enzymatic Analysis*. Academic Press, New York, 129–217 (1972).
18. Giblin, F. J., and V. N. Reddy. Pyridine nucleotides in ocular tissues as determined by the cycling assay. *Exp. Eye Res.* **31**;601–609 (1980).
19. Akerboom, J. P. M., and H. Sies. Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. *Methods Enzymol.* **77**:373–382 (1981).
20. Hassid, W. Z., and S. Abraham. Chemical procedures for analysis of polysaccharides. *Methods Enzymol* **3**:34–50 (1957).
21. Luine, V. N., and F. C Kauffman. Triphosphopyridine nucleotide-dependent enzymes in the developing spinal cord of the rabbit. *J. Neurochem.* **18**:1113–1124 (1971).
22. Hetu, C., and J.-G. Joly. Differences in the duration of the enhancement of liver mixed-function oxidase activities in ethanol-fed rats after withdrawal. *Biochem. Pharmacol.* **34**:1211–1216 (1985).
23. Villet, R. H., and K. Dalziel. The nature of the carbon dioxide substrate and equilibrium constant of the 6-phosphogluconate dehydrogenase reaction. *Biochem. J.* **115**:633–638 (1969).
24. Belinsky, S. A., L. A Reinke, R. Scholz, F. C. Kauffman, and R. G. Thurman. Rates of pentose cycle flux in perfused rat liver. Evaluation of the role of reducing equivalents from the pentose cycle for mixed-function oxidation. *Mol. Pharmacol.* **28**:371–376 (1985).
25. Winston, G. W., and R. Reitz. Effects of chronic ethanol ingestion on male and female rat liver glycogen phosphorylase phosphataše. *Alcoholism (Zagreb)* **8**:277–282 (1984).
26. Eggleston, L. V., and H. A. Krebs. Regulation of the pentose phosphate cycle. *Biochem. J.* **138**:425–435 (1974).
27. Cubero, A., F. J. Moreno, and F. Mayor. Roles of cAMP and free fatty acids on the activity of the pentose phosphate shunt. *Mol. Cell. Biochem.* **47**:175–181 (1982).
28. Ros, M., A. Cubero, M. F. Lobato, J. P. Garcia-Ruiz, and F. J. Moreno. Fatty acyl-CoA as feedback regulators of hexose monophosphate shunt in rat adipocytes. *Mol. Cell. Biochem.* **631**:119–123 (1984).
29. Belinsky, S. A., F. C. Kauffman, and R. G. Thurman. Reducing equivalents for mixed function oxidation in periportal and pericentral regions of the liver lobule in perfused livers from normal and phenobarbital-treated rats. *Mol. Pharmacol.* **26**:574–581 (1984).
30. Weigl, K., and H. Sies. Drug oxidations dependent on cytochrome P-450 in isolated hepatocytes. The role of the tricarboxylates and aminotransferases in NADPH supply, *Eur. J. Biochem.* **77**:401–408 (1977).
31. Mason, H. S. Mechanisms of oxygen metabolism. *Science (Wash. D. C.)* **125**:1185–1188 (1957).
32. Gillette, J. R., B. B. Brodie, and B. N. La Du. The oxidation of drugs by liver microsomes: on the role of TPNH and oxygen. *J. Pharmacol. Exp. Ther.* **119**:532–540 (1957).
33. Gorsky, L. D., D. R. Koop, and M. J Coon. On the stoichiometry of the oxidase and monooxygenase reactions catalyzed by liver microsomal cytochrome P-450 Products of oxygen reduction. *J. Biol. Chem.* **259**:6812–6817 (1984).
34. Jones, D. P., L. Eklow, H. Thor, and S. Orrenius. Metabolism of H_2O_2 in isolated hepatocytes: relative contributions of catalase and glutathione peroxidase in decomposition of endogenously generated H_2O_2. *Arch. Biochem. Biophys.* **210**:505–516 (1981).
35. Videla, L. A., and A. Valenzuela. Alcohol ingestion, liver glutathione and lipoperoxidation: metabolic interrelations and pathological implications. *Life Sci.* **31**:2395–2407 (1982).

Send reprint requests to: Dr. Lester A. Reinke, Department of Pharmacology, University of Oklahoma Health Sciences Center, P. O. Box 26901, Oklahoma City, OK 73190.