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INHIBITION OF MIXED-FUNCTION OXIDATION IN PERFUSED RAT LIVER BY FLUOROACETATE TREATMENT*

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Abstract—The effect of fluoroacetate, an inhibitor of the citric acid cycle, on the mixed-function oxidation of *p*-nitroanisole in isolated perfused livers from fed rats was studied. The citric acid cycle was inhibited by injection of 5 mg/kg sodium fluoroacetate into rats 3 hr prior to liver perfusion experiments. Inhibition of the citric acid cycle was marked by accumulation of citrate (5-fold) and decreases in rates of glycolysis and glycogenolysis by 50–90%. Fluoroacetate treatment inhibited mixed function oxidation in the perfused liver by about 50% without affecting *p*-nitroanisole O-demethylation by isolated microsomes. Fluorocitrate, at concentrations up to 50 μM, did not inhibit microsomal *p*-nitroanisole O-demethylation *in vitro*. These data support the hypothesis that mixed-function oxidation in intact hepatocytes is dependent upon reducing equivalents generated via the citric acid cycle.

Several studies have indicated that mixed-function oxidation is regulated in the whole cell by the supply of the cofactor NADPH [1–3]. NADPH may be formed in the cytosol via oxidation of hexose phosphates or may arise in the mitochondrial space as a result of activities such as β-oxidation and the citric acid cycle. Over two-thirds of the hepatocellular NADPH is located in the mitochondrial space: this pool is about −10 mV more reduced than the cytosolic pool [4]. Recently, Danis *et al.* [5] showed that mixed-function oxidation in perfused liver from fasted rats is strongly inhibited by 2-bromooctanoate, an inhibitor of β-oxidation. They concluded that fatty acid oxidation supplies most of the reducing equivalents for mixed-function oxidation in the fasted state. In the fed state, the source of reducing equivalents for mixed-function is unknown; however, it possibly involves the pentose cycle and/or the citric acid cycle. Furthermore, 6-aminonicotinamide abolished NADPH generation via the pentose cycle but did not affect rates of *p*-nitroanisole metabolism in the perfused liver.¶ In contrast, potassium cyanide, an inhibitor of the mitochondrial respiratory chain, decreased rates of O-demethylation significantly but did not affect rates of NADPH generation via the pentose cycle.¶ These data suggest that, even in the fed state, generation of NADPH via the pentose cycle is not obligatory for the mixed-function oxidation of xenobiotics as has been suggested previously [6].

Fluoroacetate is a potent inhibitor of the citric acid cycle due to a "lethal synthesis" of fluorocitrate which inhibits aconitase causing large accumulations of citrate [7]. Therefore, fluoroacetate was used to evaluate the role of reducing equivalents from the citric acid cycle for mixed-function oxidation in the fed state. The data are consistent with the hypothesis that fluoroacetate inhibits mixed-function oxidation by 40–50% by diminishing the supply of reducing equivalents generated in the mitochondria via the citric acid cycle.

MATERIALS AND METHODS

Animals. Female Sprague–Dawley rats, 150–300 g, were pretreated with sodium phenobarbital (1 mg/ml) in drinking water for 1–2 weeks to induce microsomal mixed-function oxidases [8]. Fluoroacetate (Sigma) was administered by intraperitoneal injection of the sodium salt in saline at a dose of 5 mg/kg 3 hr prior to the perfusion experiment. Fasted animals were deprived of food for 24 hr prior to use.

Liver perfusion. Rat livers were perfused by a method described previously [9]. Livers were perfused at 37° with Krebs–Henseleit bicarbonate buffer, pH 7.4, saturated with an oxygen–carbon dioxide mixture (95:5) in a non-recirculating system. The perfusion medium did not contain glucose. The effluent perfusate was pumped via a cannula placed in the vena cava past a teflon-shielded oxygen electrode for continuous measurement of venous oxygen tensions. Rates of oxygen uptake were calculated from the liver weight, the flow rate, and the arterial minus venous oxygen concentration differences and were used routinely to assess viability of the preparations.

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¶ S. A. Belinsky, L. A. Reinke, R. Scholz, F. C. Kauffman and R. G. Thurman, manuscript submitted for publication.

p-*Nitroanisole metabolism.* *p*-Nitroanisole (Kodak) was dissolved (0.2 mM) in Krebs–Henseleit bicarbonate buffer by heating at 55° for 1–2 hr. The formation of unconjugated *p*-nitrophenolate ion by the liver was monitored spectrally as described previously [10]. Total (free + conjugated) *p*-nitrophenol was determined by incubating a 1-ml sample of effluent perfusate collected at 2-min intervals with 25 units of sulfatase (Sigma) and 250 Fishman units of β-glucuronidase (Sigma) for 90 min at room temperature. More than 95% of the glucuronide and sulfate conjugates of *p*-nitrophenol were hydrolyzed with this procedure [11]. The *p*-nitrophenol liberated was then measured spectrally at 436 nm, and rates of *p*-nitroanisole O-demethylation were expressed as the sum of free and conjugated *p*-nitrophenol produced per gram per unit time.

Hepatic microsomal p-*nitroanisole O-demethylase activity and mitochondrial aconitase activity.* Microsomes were prepared by standard differential centrifugation. Rates of hepatic microsomal *p*-nitroanisole O-demethylase were determined by incubating microsomes in 1 ml of reaction mixture containing 0.1 M potassium phosphate buffer, pH 7.4, 0.2 mM *p*-nitroanisole, 10 mM nicotinamide, 0.1 mM dithiothreitol, 0.02% bovine serum albumin and 2 mg microsomal protein. The reaction mixture also contained 5 mM glucose-6-phosphate and 0.1 units of glucose-6-phosphate dehydrogenase/ml as an NADPH-regenerating system. Reactions were started with the addition of 1 mM $NADP^+$ and terminated after 7 min by the addition of 0.35 ml of 0.9 M perchloric acid. The mixture was centrifuged, and 1.0 ml of the supernatant fraction was mixed with 0.1 ml of 12 N NaOH to enhance the absorbance of *p*-nitrophenol, which was determined at 436 nm using the extinction coefficient of 7.11 $mM^{-1}\,cm^{-1}$ [12]. Microsomal protein was determined by the biuret reaction [13] and *p*-nitroanisole O-demethylase activity was determined from the sum of *p*-nitrophenol and 4-nitrocatechol ($\Sigma_{480} = 8.61$ $mM^{-1} \times cm^{-1}$) formed per minute per milligram of microsomal protein.

Experiments involving fluorocitrate were performed using the potassium salt prepared by incubating 1 ml of a 5 mM solution of the barium salt (Sigma Chemical Co.) at 23° for 10 min with 1 mM potassium sulfate. The barium sulfate precipitate was removed by centrifugation at 10,000 *g* for 10 min.

The influence of fluorocitrate on aconitase in mitochondria was assessed by measuring the production of isocitrate by mitochondria incubated with 1 mM citrate in 25 mM potassium phosphate buffer, pH 7.4, containing: 250 mM mannitol, 0.1 mM dithiothreitol, 0.02% bovine serum albumin and 0.5 mM $MgCl_2$. Mitochondria were isolated according to standard procedures [14], and isocitrate was measured fluorometrically [15] after 10 min of incubation.

Metabolite assays in perfusate. Glucose, lactate and pyruvate in the perfusate were assayed enzymatically by standard techniques [16]. Rates of production of metabolites were calculated from the arterial minus venous concentration differences, the flow rate, and the liver wet weight.

Livers were freeze-clamped with tongs chilled in liquid nitrogen after 28 min of perfusion [17]. Livers were extracted in perchloric acid and citrate and glucose-6-phosphate were determined in the neutralized extract by standard enzymatic techniques [15]. $NADP^+$ and NADPH were measured as described elsewhere [16].

RESULTS

Effect of fluoroacetate on hepatic citrate content. Fluoroacetate treatment increased the citrate content in freeze-clamped livers over 5-fold (Table 1). This increase of citrate was highly significant ($P < 0.001$) and confirms previous work showing that fluoroacetate inhibits the citric acid cycle [7].

Effect of fluoroacetate on p-*nitroanisole O-demethylation in perfused livers from fed rats.* When *p*-nitroanisole was infused into livers from fed, phenobarbital-treated rats, maximal rates of *p*-nitrophenol production of 6–7 $\mu moles \cdot g^{-1} \cdot hr^{-1}$ were achieved in 6–8 min (Fig. 1). Subsequently, the rate declined slightly over the next 15 min of perfusion. In contrast, maximal rates of *p*-nitrophenol production in livers from fluoroacetate-treated rats were diminished by about 50% (Fig. 1). This effect was associated with a strong tendency for the NADP/NADPH × 100 ratio to be lowered [control, 55 ± 15; fluoroacetate-treated, 92 ± 22 ($N = 5$)].

Effect of fluoroacetate treatment and fluorocitrate on p-*nitroanisole O-demethylation in isolated hepatic microsomes.* In isolated microsomes supplied with an active NADPH-generating system, fluoroacetate pretreatment had no effect on *p*-nitroanisole O-demethylation (Table 2). Addition of either fluoroacetate or fluorocitrate (up to 50 μM final concentration) to incubation mixtures also had no effect on

Table 1. Citrate content in the perfused livers from fed, phenobarbital-treated rats*

Group	No. of rats	Citrate (μmoles/kg liver wet wt)
Control	6	84 ± 22
Fluoroacetate-treated	4	447 ± 56†

* Treated rats were injected i.p. with 5 mg/kg of sodium fluoroacetate 3 hr prior to perfusion. Livers from both treated and control groups were freeze-clamped with tongs chilled in liquid nitrogen after 28 min of perfusion. Citrate was determined by standard enzymatic techniques. Values are expressed as mean ± S.E.M.

† $P < 0.001$ with respect to control value.

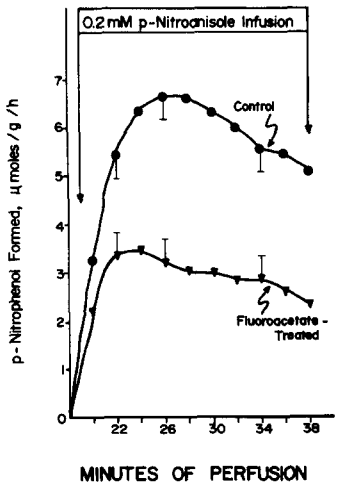

Fig. 1. Effect of fluoroacetate treatment on *p*-nitroanisole O-demethylation in perfused liver. Fed, phenobarbital-treated rats were treated with fluoroacetate (5 mg/kg) or vehicle 3 hr prior to perfusion. *p*-Nitroanisole (0.2 mM) was infused as indicated by the horizontal bar. Mean of nine experiments. Vertical bars represent S.E.M.

microsomal *p*-nitroanisole O-demethylase activity (Table 3). If fluoroacetate is distributed uniformly in the water space, concentrations would not exceed 75 μM. Since liver perfusions were performed 3 hr after administering fluoroacetate, it is highly likely that hepatic concentrations of newly synthesized fluorocitrate were considerably less than 50 μM. Thus, inhibition of *p*-nitrophenol formation noted in the intact liver of fluoroacetate-treated animals cannot be ascribed to direct effects of fluorocitrate on the mixed-function oxidase system.

In contrast to livers from fed rats, fluoroacetate treatment had no effect on *p*-nitroanisole O-demethylation in livers from fasted rats (Fig. 2). The subsequent infusion of xylitol, a substrate for NADPH generation, increased mixed-function oxidation 2–3 $\mu moles \cdot g^{-1} \cdot hr^{-1}$ in livers from both control and fluoroacetate-treated rats (Fig. 2).

Effect of fluoroacetate on rates of production of glucose, lactate and pyruvate by the perfused liver. Figure 3 shows the mean rate of glucose production in livers from control and fluoroacetate-treated rats.

Table 3. Effect of added fluoroacetate and fluorocitrate on microsomal *p*-nitroanisole O-demethylase *in vitro**

Addition	*p*-Nitroanisole O-demethylase [$nmoles \cdot (mg\ protein)^{-1} \cdot min^{-1}$]
None	2.61 ± 0.08
Fluoroacetate	
5 μM	2.66 ± 0.10
50 μM	2.64 ± 0.03
Fluorocitrate	
5 μM	2.55 ± 0.03
50 μM	2.70 ± 0.05

* Values are averages ± S.E.M. of three to six replicate samples. Microsomes were isolated from a phenobarbital-treated rat and incubated at 30° for 7 min as described in Materials and Methods.

Fluoroacetate treatment decreased glucose output by 85–90% (Fig. 3). Glycolysis, as reflected by lactate plus pyruvate production, was also diminished over 50% by treatment with fluoroacetate (Fig. 4). In addition, contents of glucose-6-phosphate were reduced from 206 ± 108 to 55 ± 16 μmoles/kg wet weight by treatment with fluoroacetate.

DISCUSSION

Effect of fluoroacetate on rat liver. One of the most characteristic actions of fluoroacetate on tissue is the accumulation of citrate due to the lethal synthesis of fluorocitrate and inhibition of aconitase leading to inhibition of the citric acid cycle [7, 18, 19]. In our study, this was marked by a striking accumulation of citrate (447 μmoles/kg wet tissue, Table 1) 3 hr after the injection of 5 mg/kg sodium fluoroacetate. Lethal fluoroacetate poisoning also causes marked depletion of hepatic glycogen [7]. One hour after fluoroacetate injection, the glycogen level was reduced by 75%, and after 2 hr it was depleted to more than 90% of control levels [18]. Although rates of glucose production (Fig. 3) and glycolysis (Fig. 4) were reduced markedly in livers 3 hr after treatment of the rat with fluoroacetate, sufficient substrate remained to maintain citrate at elevated levels during perfusion experiments. The decreases in rates of glucose and lactate plus pyruvate production in all likelihood reflect depletion of carbohydrate reserves by fluoroacetate as well as by the well known

Table 2. Effect of fluoroacetate on *p*-nitroanisole O-demethylase activity in hepatic microsomes from fed, phenobarbital-treated rats*

Group	No. of rats	*p*-Nitroanisole O-demethylase activity [nmoles product formed $\cdot min^{-1}$] $\cdot$ (mg microsomal protein)$^{-1}$]
Control	4	2.10 ± 0.11
Fluoroacetate-treated	4	1.92 ± 0.08

* Values are mean ± S.E.M. of three replicate samples. The differences between the two groups were not satistically significant. Microsomal protein was determined by the biuret reaction. *p*-Nitroanisole O-demethylase activity was determined from the sum of *p*-nitrophenol and 4-nitrocatechol formed per minute per milligram of microsomal protein. Rats were treated as described in the legend of Fig. 1.

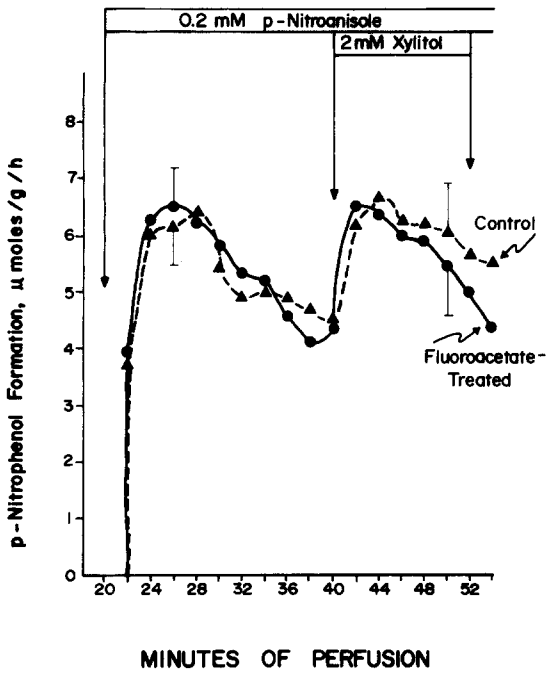

Fig. 2. Effect of fasting and xylitol on *p*-nitroanisole O-demethylation in perfused liver from fluoroacetate-treated rats. Fasted, phenobarbital-treated rats were treated with fluoroacetate as described in Materials and Methods. Infusion of *p*-nitroanisole (0.2 mM) and of xylitol (2 mM) is indicated by the horizontal bars. Mean of four experiments. Vertical bars represent S.E.M.

inhibition of glycolysis by citrate and fluorocitrate [7].

Effect of fluoroacetate on hepatic mixed-function oxidation. The experiments reported here show clearly that the O-demethylation of *p*-nitroanisole

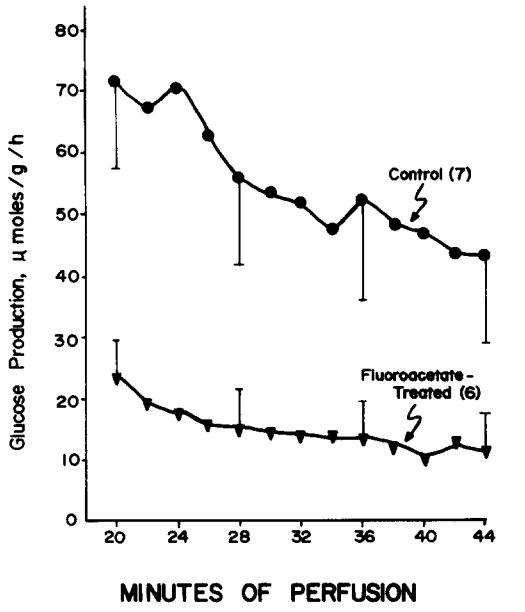

Fig. 3. Effect of fluoroacetate on the rate of glucose production. Livers of fed, phenobarbital-treated rats were perfused under conditions as in Fig. 1.

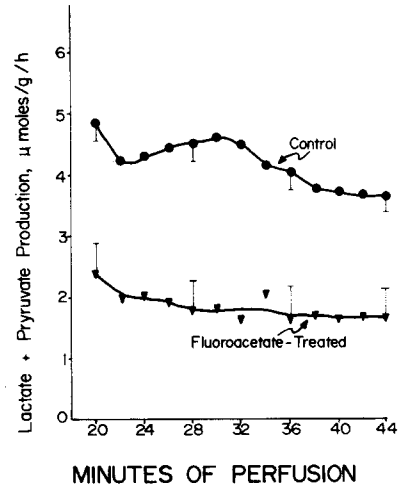

Fig. 4. Effect of fluoroacetate lactate plus pyruvate productions. Conditions as in Fig. 1.

was inhibited by about 50% (Fig. 1) 3 hr after pretreatment with fluoroacetate. The mechanism of this inhibition does not involve direct inhibition of microsomal mixed-function oxidation by fluoroacetate or newly synthesized fluorocitrate. Addition of either fluorocitrate or fluoroacetate to microsomes *in vitro* had no effect on *p*-nitroanisole O-demethylase (Table 3). The possibility that fluoroacetate treatment altered the mixed-function oxidases of the endoplasmic reticulum *in vivo* can also be ruled out since isolated microsomes from fluoroacetate-treated rats oxidized *p*-nitroanisole at rates similar to microsomes from control rats when incubated with excess NADPH (Table 2). Therefore, an effect of fluoroacetate treatment on NADPH supply must be considered. In livers from fed rats, NADPH may be supplied from oxidation of hexose phosphates via the pentose cycle or via the oxidation of acetyl CoA by the citrate cycle. Mixed-function oxidation in livers from both control and fluoroacetate-treated rats appears to be limited by NADPH supply because xylitol stimulated *p*-nitroanisole O-demethylation (Fig. 3) [20]. This hypothesis is supported by the observation that fluoroacetate treatment tended to decrease the $NADP^+/NADPH \times 100$ ratio strongly ($P < 0.1$ but greater than 0.05).

Inhibition of mixed-function oxidation by fluoroacetate in perfused liver may be due to either: (a) direct inhibition of the citric acid cycle and decreased generation of reducing equivalents, or (b) depletion of glycogen and substrate for the formation of reducing equivalents via the pentose phosphate pathway. Several facts support the hypothesis that the citric acid cycle is a primary source of reducing equivalents for mixed-function oxidation. First, fluoroacetate treatment inhibited mixed-function oxidation in the intact liver and greatly elevated hepatic citrate levels. Presumably this effect results in a decrease in either the generation of reducing equivalents or intermediates required for substrate shuttle mechanisms to move mitochondrial reducing equivalents into the cytosol [21]. Second, inhibition of phosphofructokinase by citrate [22] could increase glucose-6-phosphate [23] and presumably increase flux through the

pentose phosphate pathway; however, the observation that glucose-6-phosphate levels were decreased by fluoroacetate allows this hypothesis to be rejected. Glycogen reserves were depleted after fluoroacetate treatment, and rates of lactate and glucose production were reduced markedly (Figs. 3 and 4).

In livers from fasted rats, fluoroacetate did not inhibit mixed-function oxidation (Fig. 3); however, the nutritional state of the rat is an important determinant in fluoroacetate toxicity. Fed animals accumulated large amounts of citrate, whereas the accumulation in fasted rats has been reported to be slight [19]. Thus, fluoroacetate may not have blocked the citric acid cycle in livers from fasted rats in this study. On the other hand, Danis *et al.* [5] have showed that reducing equivalents for mixed-function oxidation arise nearly totally via β-oxidation of acyl CoA compounds in the fasted state. One would not expect fluoroacetate treatment to affect this process.

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