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p-NITROANISOLE O-DEMETHYLATION IN PERFUSED HAMSTER LIVER

HIGH RATES OF PENTOSE CYCLE-INDEPENDENT MIXED-FUNCTION OXIDATION*

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Abstract—Rates of *p*-nitroanisole O-demethylation in perfused livers from Syrian golden hamsters were three to four times greater than comparable rates measured in preparations from Sprague–Dawley rats. Hamsters also had greater microsomal *p*-nitroanisole *O*-demethylase activity and cytochrome P-450 contents than rats. In general, phenobarbital caused similar increases in these properties in both species. Fasting of hamsters for 24 hr increased *p*-nitroanisole *O*-demethylase activity in microsomes but did not affect rates in perfused livers. Rates were also unaffected in the perfused liver by pretreatment with 6-aminonicotinamide, an inhibitor of the pentose phosphate shunt. Hamster livers had low activities of pentose cycle enzymes but high activities of malic enzyme and isocitrate dehydrogenase compared to rats. In hamster livers, maximal rates of *p*-nitroanisole O-demethylation were not maintained but declined steadily over 40 min with prolonged *p*-nitroanisole infusion. The decreased rates of mixed-function oxidation in the non-recirculating perfusion system could not be explained by diminished tissue viability or degradation of cytochrome P-450 but were likely due to a decline in the formation of reduced cofactor. Hepatic concentrations of α-ketoglutarate and malate increased during *p*-nitroanisole infusion. Furthermore, rates of *p*-nitroanisole O-demethylation were inhibited by ethanol and aminooxyacetate, agents which inhibit the generation and/or movement of mitochondrial reducing equivalents into the cytosol. The infusion of pyruvate stimulated *p*-nitroanisole O-demethylation in perfused livers from fasted hamsters. This effect was maximal with 0.1 mM pyruvate, did not require gluconeogenesis, and was insensitive to 6-aminonicotinamide treatment. Thus, stimulation of *p*-nitroanisole metabolism by pyruvate in hamster livers is likely related to the mitochondrial oxidation of pyruvate, rather than to increased NADPH generation via the pentose phosphate cycle. These data indicate that mitochondrial sources of NADPH supply reducing equivalents for mixed-function oxidation in hamster liver.

Syrian golden hamsters are popular models in cancer research because they exhibit low incidences of spontaneous tumor formation and higher susceptibility to chemical carcinogens than rats [1]. However, very little information exists concerning the biochemical basis of the increased sensitivity of hamsters to cancer-causing chemicals. One fundamental process related to chemical carcinogenesis is mixed-function oxidation. Most carcinogenic chemicals are precarcinogens that require activation via mixed-function oxidation before they are capable of initiating neoplastic changes [2]. Therefore, we have compared rates of mixed-function oxidation in perfused hamster and rat livers utilizing *p*-nitroanisole as a model substrate. The perfused liver was chosen because mixed-function oxidation studied in microsomes does not take important regulating factors, such as the supply of NADPH, into account [3–6]. *p*-Nitroanisole is a convenient substance to study in the perfused liver because the generation of *p*-nitrophenol via mixed-function oxidation can be easily monitored in the effluent perfusate [6].

The data indicate that rates of *p*-nitroanisole O-demethylation were nearly 4-fold greater in hamster than in rat livers and that mitochondria are important sources of NADPH for *p*-nitroanisole metabolism in hamster livers.

MATERIALS AND METHODS

Animals. Male Syrian golden hamsters, 95–105 g, were utilized in all experiments. Fed hamsters had access to lab chow *ad lib.* and fasted animals were deprived of food for 24 hr prior to use. Where indicated, animals received sodium phenobarbital (1 mg/ml) in drinking water for 7–10 days prior to perfusion experiments to induce mixed-function oxidase activity [7]. Sprague–Dawley rats treated in the same manner were utilized in comparative studies.

Liver perfusion and determination of p-*nitrophenol.* Details of the non-recirculating perfusion technique have been described elsewhere [8]. Livers

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were perfused with Krebs–Henseleit bicarbonate buffer, pH 7.4, 37°, which was saturated with an oxygen–carbon dioxide mixture (95:5). The perfusate was pumped via a cannula placed in the vena cava past a Teflon-covered, Clark-type oxygen electrode so that oxygen uptake could be monitored continuously to assess tissue viability. *p*-Nitroanisole (0.2 mM) was dissolved in the buffer and production of *p*-nitrophenolate ion was monitored spectrally at 436 nm as described previously [6]. Under these conditions, formation of 4-nitrocatechol from *p*-nitrophenol was minimal. Sulfate and glucuronide conjugates of *p*-nitrophenol were measured by incubating 1.0 ml of perfusate with 0.1 ml of 0.5 M Tris–HCl buffer, pH 7.4, containing 250 units of β-glucuronidase and 25 units of sulfatase (Sigma) for 90 min at room temperature. Pyruvate, ethanol, glucose and aminooxyacetate were dissolved in the buffer and infused into livers at the final concentrations shown in the text and figure legends.

Hepatic microsomal p*-nitroanisole* O*-demethylase activity and cytochrome P-450 content.* Hepatic microsomes were prepared by standard techniques of differential centrifugation [9]. They were subsequently washed and resuspended in 0.15 M KCl. *p*-Nitroanisole *O*-demethylase activity was assayed in 25-ml Erlenmeyer flasks containing 180 mM phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 0.5 mM *p*-nitroanisole, microsomes (2–3 mg/ml), and an NADPH-generating system consisting of 0.4 mM $NADP^+$, 30 mM DL-isocitrate, and 0.2 units isocitrate dehydrogenase (Sigma) in a final incubation volume of 2.0 ml. Incubations were initiated by the addition of the NADPH-generating system and were terminated after 15 min by the addition of 0.5 ml of 0.6 N perchloric acid. The precipitated microsomal protein was removed by centrifugation, and 1.0 ml of supernatant fraction was mixed with 0.1 ml of 12 N NaOH. *p*-Nitrophenol and 4-nitrocatechol were determined spectrally [10]. *p*-Nitroanisole *O*-demethylase activity was expressed as the sum of *p*-nitrophenol and 4-nitrocatechol formed $\cdot$ min^{-1} $\cdot$ (mg microsomal protein)$^{-1}$ as described elsewhere [11]. The biuret method was used to measure microsomal protein [12]. Microsomal cytochrome P-450 was determined employing an Aminco DW2A split-beam spectrophotometer and an extinction coefficient of 91 mM^{-1} [13].

Measurement of metabolic intermediates. Metabolic intermediates were measured in $HClO_4$ extracts of livers that had been frozen with tongs chilled in liquid nitrogen. Samples of frozen liver weighing about 200 mg were powdered and extracted with 0.3 M $HClO_4$ as described previously [14]. The protein-free extracts were neutralized with 2 M $KHCO_3$ and stored at −80° until assayed for metabolites. Concentrations of glucose and lactate in perfusate were measured enzymatically [15].

Determination of enzyme activities. Enzyme activities were determined in samples of livers that were homogenized in 10 vol. of 0.25 M sucrose containing 2 mM $CaCl_2$ and 5 mM sodium phosphate, pH 7.0. All enzyme assays were performed fluorimetrically at room temperature by measuring initial rates of oxidation or reduction of $NADP^+$ or NADPH after addition of appropriate substrates. "Malic" enzyme (EC 1.1.1.38), isocitrate dehydrogenase (EC 1.1.1.42), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.43) activities were determined as described previously [16].

RESULTS

Comparison of rates of p*-nitroanisole O-demethylation in perfused livers from hamsters and rats.* Following 20 min of preperfusion with Krebs–Henseleit bicarbonate buffer, the infusion of *p*-nitroanisole into a liver from a fed hamster led to a rapid production of free and conjugated *p*-nitrophenol (Fig. 1, left panel). The maximal rate of *p*-nitroanisole O-demethylation was 11.7 μmoles *p*-nitrophenol formed $\cdot$ g^{-1} $\cdot$ h^{-1}. However, the hamster liver maintained this high rate of metabolism for only 1–2 min. Subsequently, the rate declined to about 40% of the maximal rate during an additional 30 min of perfusion. The maximal rate of *p*-nitroanisole metabolism in a perfused liver from a fed, normal rat was much lower (3 μmoles $\cdot$ g^{-1} $\cdot$ hr^{-1}). In contrast, however, the rate did not decline markedly over the next 20–30 min of *p*-nitroanisole infusion (Fig. 1, right panel).

The effects of fasting and phenobarbital treatment on maximal rates of *p*-nitroanisole O-demethylation and cytochrome P-450 concentrations in hamster and rat livers are compared in Table 1. Rates of *p*-nitroanisole metabolism and cytochrome P-450 content were higher in perfused livers and microsomes from hamsters than corresponding values observed with rats. Fasting did not affect cytochrome P-450 content or rates of *p*-nitroanisole metabolism in perfused livers or microsomes from rats. Fasting increased both *p*-nitroanisole *O*-demethylase activity (56%) and cytochrome P-450 content (28%) in hamster microsomes but did not affect rates of mixed-function oxidation in perfused hamster livers (Table 1).

Phenobarbital treatment increased rates of *p*-nitroanisole metabolism by 3- to 4-fold in perfused livers and isolated microsomes from both hamsters and rats (Table 1); however, concentrations of cytochrome P-450 were similar in microsomes from both species after phenobarbital treatment (Table 1).

Activity of NADPH-generating enzymes in hamster and rat liver. The activities of several NADPH-generating enzymes were measured in homogenates of hamster and rat liver (Table 2). The activity of the enzymes of the pentose phosphate shunt (glucose-6-phosphate and 6-phosphogluconate dehydrogenase) were 2- to 3-fold lower in livers of hamsters than rats. Conversely, activities of enzymes which derive their substrates from mitochondrial sources ("malic" enzyme and isocitrate dehydrogenase) were nearly twice as high in hamster as in rat liver.

Changes in cellular intermediates during mixed-function oxidation in perfused hamster livers. In livers from fed, untreated hamsters, rates of *p*-nitrophenol formation from *p*-nitroanisole were maximal approximately 6 min after initiation of *p*-nitroanisole infusion but subsequently declined to approximately 50% of maximum after 30 min of continued substrate infusion (Fig. 1). To gain understanding of factors

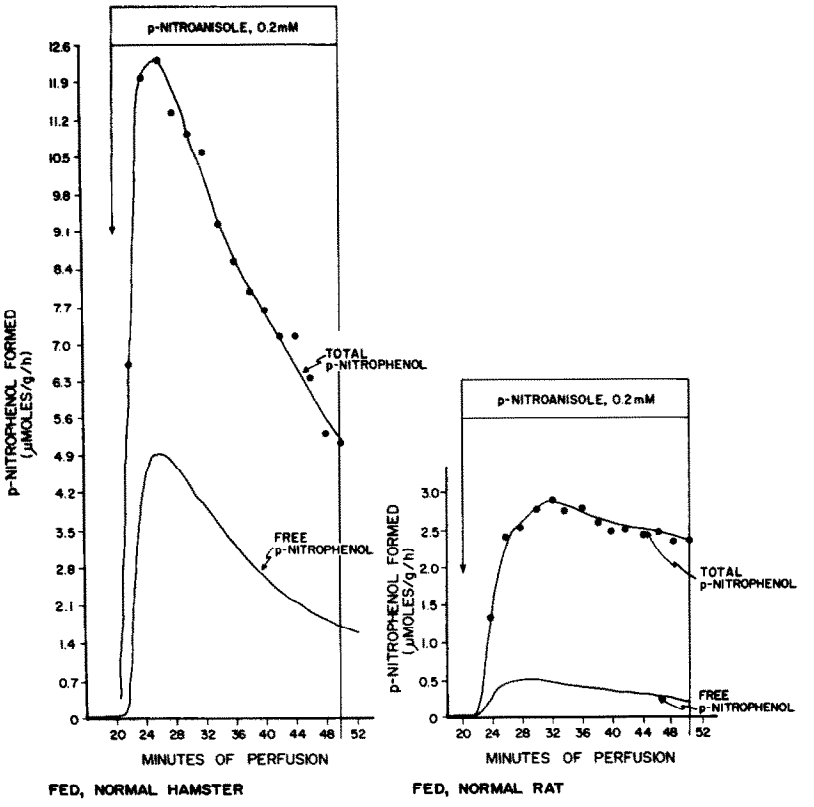

Fig. 1. *p*-Nitrophenol formation from *p*-nitroanisole in perfused livers from hamsters and rats. After 20 min of preperfusion with Krebs–Henseleit bicarbonate buffer, *p*-nitroanisole (0.2 mM) was infused during the times designated by the horizontal bars and vertical arrows. The release of free, nonconjugated *p*-nitrophenol was monitored spectrally in the effluent perfusate [6]. Samples of perfusate (1.0 ml) were collected every 2 min and were incubated with glucuronidase and sulfatase as described in Materials and Methods. The total rate (●—●) of production of *p*-nitrophenol (free + conjugated) was superimposed above the continuous record at the appropriate time points. Left panel: untreated, fed hamster. Right panel: untreated, fed rat. Apparent differences in the proportion of *p*-nitrophenol conjugated between species and with prolonged perfusion time are most likely the result of different intracellular concentrations of *p*-nitrophenol formed from *p*-nitroanisole [10].

Table 1. Effects of fasting and phenobarbital treatment on cytochrome P-450 concentration and rates of *p*-nitroanisole O-demethylation in perfused livers and isolated microsomes from hamsters and rats

Nutritional and induction state	Maximal rate of *p*-nitroanisole metabolism in perfused livers* (μmoles/g wet wt/hr)		Microsomal *p*-nitroanisole *O*-demethylase activity† (nmoles/mg protein/min)		Cytochrome P-450 concentration† (nmomes/mg protein)	
	Hamster	Rat	Hamster	Rat	Hamster	Rat
Fed, normal	13.2 ± 0.7‡	2.4 ± 0.3	0.77 ± 0.08‡	0.40 ± 0.03	1.06 ± 0.06‡	0.71 ± 0.06
Fasted, normal	12.5 ± 0.8‡	3.0 ± 0.3	1.20 ± 0.01‡§	0.43 ± 0.09	1.36 ± 0.02‡§	0.65 ± 0.07
Fed, phenobarbital-treated	37.2 ± 5.5‡§	8.6 ± 1.1§	2.41 ± 0.20‡§	1.41 ± 0.13§	2.03 ± 0.12§	2.10 ± 0.11§

* Maximal rates of *p*-nitrophenol formation occurred approximately 6 min after *p*-nitroanisole infusion was initiated (see Fig. 1). Values are free + conjugated *p*-nitrophenol from five to sixteen livers per group ± S.E.M.

† *p*-Nitroanisole *O*-demethylase activity and cytochrome P-450 content were measured as described in Materials and Methods. Each value is the mean ± S.E.M. from at least four animals. Recovery of microsomal protein was similar in both species.

‡ $P < 0.001$ compared to corresponding values from rats.

§ $P < 0.05$ compared to corresponding values from fed, normal animals of the same species, using Student's *t*-test.

Table 2. Comparison of the activities of NADPH-generating enzymes in liver homogenates from hamsters and rats*

	Glucose-6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase (μmoles/g protein/hr)	"Malic" enzyme	Isocitrate dehydrogenase
Hamster	2.3 ± 0.1†	3.8 ± 0.1†	5.1 ± 0.4†	120.2 ± 3.2†
Rat	5.0 ± 0.6	12.3 ± 0.9	3.6 ± 0.7	79.6 ± 6.6

* Enzyme activities were measured in liver homogenates as described in Materials and Methods. Each value is the mean ± S.E.M. from sixteen hamsters or eight rats.
† $P < 0.05$ that the difference between hamsters and rats would occur by chance.

responsible for this decline, livers were freeze-clamped during maximal rates of *p*-nitroanisole metabolism or after the rates had declined by more than 50% (6 and 30 min after initiation of *p*-nitroanisole infusion respectively), and concentrations of various metabolites were determined.

Concentrations of intermediates of the pentose pathway including glucose-6-phosphate, xylulose-5-phosphate, ribulose-5-phosphate, and 6-phosphogluconate were not altered nor were isocitrate, lactate, ATP and ADP after 30 min of *p*-nitroanisole infusion (Table 3). In contrast, concentrations of α-ketoglutarate and malate were increased nearly 2-fold over the same time course (Table 3).

Effects of ethanol and aminooxyacetate on p-*nitroanisole O-demethylation in perfused livers from fed hamsters.* When ethanol (20 mM) was infused into a liver from a fed hamster, the rate of *p*-nitroanisole metabolism was inhibited approximately 70% (Fig. 2). The inhibition reversed rapidly when ethanol infusion was discontinued. The subsequent infusion of aminooxyacetate (0.5 mM), a transaminase inhibitor [17], also diminished rates of *p*-nitroanisole metabolism by about 40% (Fig. 2). At these concentrations, ethanol and aminooxyacetate inhibited microsomal *p*-nitroanisole metabolism by less than 20 and 10% respectively (data not shown).

Effects of glucose and pyruvate on p-*nitroanisole O-demethylase activity in perfused livers from fasted hamsters.* The effects of 0.1 and 1.0 mM pyruvate on rates of *p*-nitroanisole O-demethylation, gluconeogenesis, and lactate production are shown in Fig. 3. Both 0.1 and 1.0 mM pyruvate increased rates of *p*-nitrophenol formation from *p*-nitroanisole 1.2 to 1.3 $\mu moles \cdot g^{-1} \cdot hr^{-1}$. The infusion of 0.1 mM pyruvate caused an increase in the rate of lactate production without any detectable change in glucose production (Fig. 3). In contrast, 1.0 mM pyruvate caused a larger increase in the rate of lactate release followed by a slow increase of glucose production beginning 4–6 min later (Fig. 3).

The infusion of glucose (10 mM) into a liver from a fasted hamster decreased rates of free *p*-nitrophenol production (Fig. 4) but had no effect on the total amount of *p*-nitrophenol measured after hydrolysis of the conjugates. This increase in *p*-nitrophenol

Table 3. Effect of *p*-nitroanisole infusion on hepatic concentrations of selected intermediates in perfused livers from untreated, fed hamsters*

	Hepatic concentrations of intermediates (μmoles/kg wet liver wt)	
	Minutes after *p*-nitroanisole infusion	
Intermediate	6	30
Glucose-6-phosphate	212 ± 42	167 ± 17
6-Phosphogluconate	69 ± 13	64 ± 8
Ribulose-5-phosphate	25 ± 9	53 ± 14
Xylulose-5-phosphate	20 ± 12	14 ± 6
Lactate	844 ± 142	894 ± 146
Pyruvate	72 ± 7	59 ± 12
Isocitrate	4.3 ± 0.3	5.8 ± 0.6
α-Ketoglutarate	82 ± 11	144 ± 7†
Malate	375 ± 74	682 ± 133‡
ATP	2188 ± 297	2224 ± 89
ADP	1302 ± 60	1206 ± 45

* Values are from four to five livers and are expressed as mean ± S.E.M. All livers were infused with *p*-nitroanisole (0.2 mM) after 20 min of preperfusion with Krebs–Henseleit bicarbonate buffer. Livers were freeze-clamped with tongs chilled in liquid nitrogen 6–30 min after the onset of *p*-nitroanisole infusion, and metabolites were measured as described in Materials and Methods.
† $P < 0.05$ that the difference between the 6 and the 30 min time point would occur by chance, using Student's *t*-test.
‡ $P < 0.10$ that the difference between the 6 and the 30 min time point would occur by chance, using Student's *t*-test.

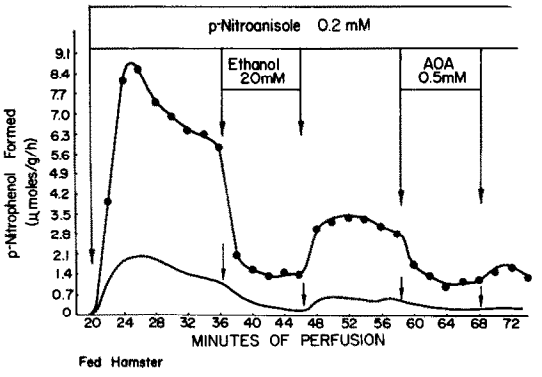

Fig. 2. Inhibition of *p*-nitroanisole O-demethylation by ethanol and aminooxyacetate in a perfused liver from a fed hamster. Infusion of *p*-nitroanisole (0.2 mM), ethanol (20 mM) and aminooxyacetate (AOA, 0.5 mM) are designated by the horizontal bars and vertical arrows. Other conditions are as given in the legend of Fig. 1.

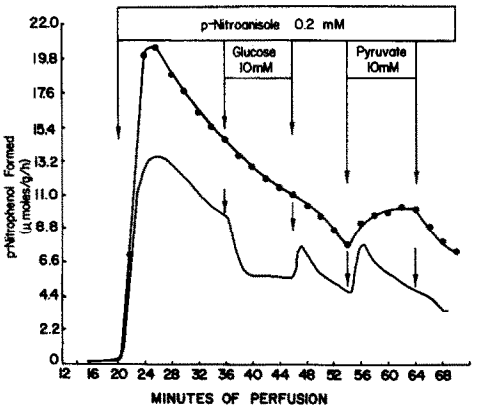

Fig. 4. Effect of glucose and pyruvate on *p*-nitroanisole metabolism in a perfused liver from a fasted hamster. The infusion of glucose (10 mM) and pyruvate (10 mM) are designated by the horizontal bars and vertical arrows. Although the rate of *p*-nitroanisole metabolism was higher than usual in this experiment, the effects of glucose and pyruvate were reproducible.

conjugation is most likely due to increased UDP-glucuronic acid formation from exogenously added glucose [10]. The subsequent infusion of pyruvate (10 mM) caused a 20–25% increase in the rate of *p*-nitrophenol formation (Fig. 4).

The effects of pyruvate (10 mM) and glucose (10 mM) on *p*-nitroanisole O-demethylation were studied in a liver of a fasted hamster 6 hr after treatment with 6-aminonicotinamide (70 mg/kg, i.p.). This dose of 6-aminonicotinamide has been shown previously to cause greater than 90% inhibition of the pentose phosphate cycle in perfused rat livers [18], which is attributed to the high affinity of 6-phosphogluconate dehydrogenase for the 6-amino $NADP^+$ analog formed after injection of this drug [19]. Maximal rates of *p*-nitroanisole O-demethylation were unaffected by pretreatment with 6-aminonicotinamide (compare Figs. 4 and 5). Under these conditions, glucose had no effect but pyruvate stimulated mixed-function oxidation (Fig. 5).

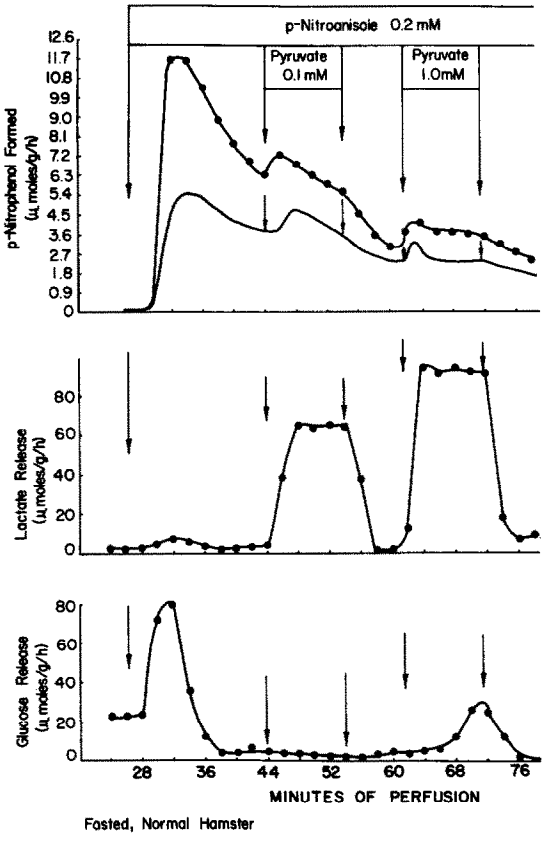

Fig. 3. Stimulation of *p*-nitroanisole O-demethylation in a perfused liver from a fasted hamster by pyruvate. The infusions of pyruvate (0.1 and 1.0 mM) are designated by the horizontal bars and vertical arrows. Concentrations of lactate (middle panel) and glucose (lower panel) in effluent perfusate were measured enzymatically [15]. The transient release of glucose after infusion of *p*-nitroanisole was typical in experiments with hamsters.

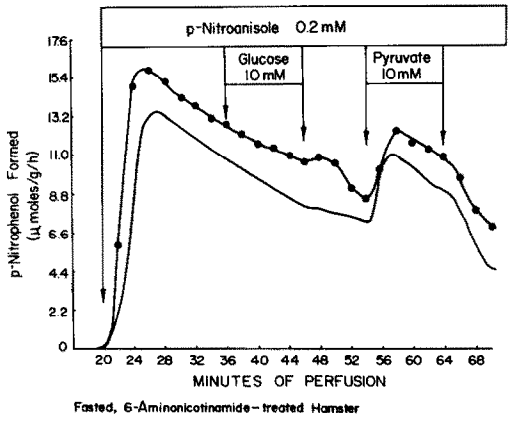

Fig. 5. Stimulation of *p*-nitrophenol formation from *p*-nitroanisole by pyruvate in a fasted hamster pretreated with 6-aminonicotinamide. A fasted hamster was pretreated with 6-aminonicotinamide (70 mg/kg, i.p.) 6 hr prior to the perfusion experiment. Other conditions are as in Fig. 4.

DISCUSSION

Comparison of rates of mixed-function oxidation in perfused livers and hepatic microsomes from hamsters and rats. *p*-Nitroanisole *O*-demethylase activity and cytochrome P-450 content were higher in hepatic microsomes from hamsters than from rats (Table 1). This increased enzyme activity in hamster livers was associated with higher rates of mixed-function oxidation in perfused hamster livers (Fig. 1, Table 1). Presumably, the higher cytochrome P-450 content in the hamster could be responsible for higher rates of mixed-function oxidation observed in the hamster. However, several lines of evidence indicate that higher rates of mixed-function oxidation by perfused livers from hamsters cannot be explained solely on the basis of differing microsomal enzyme activities. Rates of *p*-nitroanisole metabolism were four to five times higher in livers from hamsters than from rats (Table 1, Fig. 1), but the differences in microsomal activity were only 2-fold. Second, rates of *p*-nitrophenol formation declined steadily during mixed-function oxidation in livers from untreated hamsters, but not untreated rats (Fig. 1), most likely because rates of NADPH utilization exceed rates of synthesis (see below). Finally, fasting of hamsters increased microsomal *p*-nitroanisole *O*-demethylase activity and cytochrome P-450 content (Table 1) but did not affect rates of *p*-nitroanisole metabolism by perfused livers. Collectively, these data support the hypothesis that the supply of NADPH, which is an important rate-determining factor for mixed-function oxidation in intact cells [3–6], also contributes to the differences in rates of *p*-nitroanisole metabolism observed between perfused livers from hamsters and rats. For this reason, additional experiments were designed to explore sources of NADPH generation during mixed-function oxidation in perfused hamster livers.

Kinetics of p*-nitroanisole metabolism in hamster liver.* Approximately 6 min after *p*-nitroanisole infusion to hamster liver, rates of *p*-nitrophenol production declined steadily (Figs.1–5) even though oxygen was supplied continually to the liver at concentrations 1 to 2 orders of magnitude above K_m values. Because rates could be increased markedly by phenobarbital treatment, it is also safe to conclude that substrate supply was in excess (Table 1). This decrease in activity cannot be explained by diminished cell viability because: (1) oxygen was taken up at constant rates (not shown); (2) adenine nucleotides were unaffected by prolonged *p*-nitroanisole infusion (Table 3); and (3) the livers remained capable of synthesizing glucose from pyruvate, an energy requiring process (Fig. 3). Similarly, it is unlikely that this decline in the rate of mixed-function oxidation could result from cytochrome P-450 degradation, since it can be reversed partially by pyruvate (Figs. 3–5). Thus, it is likely that the decline in rate is due to decreased generation of NADPH.

The pentose phosphate shunt is assumed to be a major source of reducing equivalents for drug metabolism in the fed state [20]. However, in experiments with fed hamsters, hepatic levels of pentose shunt and glycolytic intermediates remained constant as the rate of mixed-function oxidation declined (Table 3). Furthermore, inhibition of the pentose shunt with 6-aminonicotinamide had no effect on rates of *p*-nitroanisole O-demethylation in perfused hamster liver (Fig. 5; similar data were obtained with fed hamsters). In contrast, concentrations of α-ketoglutarate and malate, intermediates involved in NADPH generation from mitochondrial sources (Fig. 6), increased with prolonged *p*-nitroanisole infusion. The explanation for this accumulation in hamster livers is presently unknown; however, as rates of *p*-nitroanisole metabolism declined, intermediates of the citric acid cycle, not the pentose shunt, accumulated in the liver (Table 3). Thus, these data suggest that the decline in rate is related to inadequate generation of NADPH which is dependent on mitochondrial oxidations.

We have demonstrated recently that inhibition of *p*-nitroanisole O-demethylation in perfused rat livers by ethanol requires its metabolism to acetaldehyde

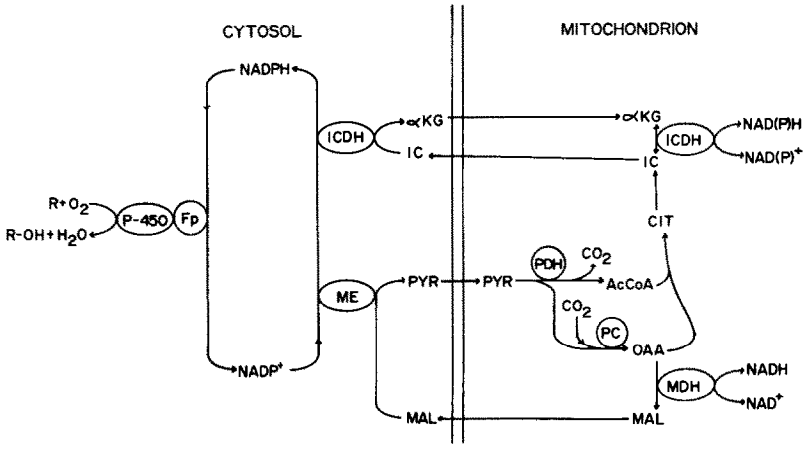

Fig. 6. Pathways of pyruvate metabolism leading to NADPH generation. Abbreviations: P-450, cytochrome P-450; Fp, NADPH cytochrome P-450 reductase; ICDH, isocitrate dehydrogenase; ME, "malic" enzyme; PYR, pyruvate; IC, isocitrate; αKg, α-ketoglutarate; MAL, malate; PDH, pyruvate dehydrogenase; PC, pyruvate carboxylase; CIT, citrate; AcCoA, acetyl CoA; OAA, oxaloacetate; and MDH, malate dehydrogenase.

[4, 21]. Inhibition by ethanol occurred concurrently with an increased $NADP^+/NADPH$ ratio [21]. Taken together, these data suggest that ethanol inhibits mixed-function oxidation in perfused livers by interfering with a mitochondrial source of NADPH. Thus, the large (70%) inhibition of *p*-nitroanisole metabolism by ethanol in perfused hamster livers (Fig. 2) is in accord with the idea that mitochondria have a significant role in generating reducing equivalents for hepatic drug metabolism in this species. This hypothesis is further supported by the observation that aminooxyacetate (Fig. 2), which interferes with NADPH biosynthesis by diminishing the transfer of reducing equivalents between mitochondria and cytosol [22], also inhibited hamster liver mixed-function oxidation.

Stimulation of p-*nitroanisole O-demethylation in perfused hamster liver by pyruvate.* Several lines of evidence support the hypothesis that reducing equivalents of mitochondrial origin are the major driving force for mixed-function oxidation in perfused hamster liver (Fig. 6). First, enzymes of the pentose phosphate cycle were low whereas mitochondrial enzymes were high in hamster compared to rat liver (Table 2). Second, 6-aminonicotinamide, an inhibitor of the pentose shunt, had no effect on either maximal rates or the kinetics of *p*-nitroanisole O-demethylation in perfused hamster liver. In contrast, ethanol and aminooxyacetate, agents which interrupt transport of mitochondrial NADPH into the cytosol, both inhibited hamster liver mixed-function oxidation (Fig. 2). To test the hypothesis that mitochondria provide reducing equivalents for mixed-function oxidation in hamster liver, we employed the mitochondrial substrate pyruvate. Stimulation of *p*-nitroanisole metabolism by pyruvate in livers from fasted hamsters does not require the prior synthesis of glucose from pyruvate. First, pyruvate stimulated *p*-nitrophenol formation in the absence of glucose synthesis (Fig. 3). Second, the increases in rates of *p*-nitrophenol formation observed during pyruvate (1.0 mM) infusion preceded the production of glucose by the liver (Fig. 3). Third, pyruvate stimulated *p*-nitroanisole metabolism in a liver where the prior infusion of glucose did not (Fig. 4). Finally, the stimulatory action of pyruvate was unaffected by treatment of hamsters with the pentose shunt inhibitor 6-aminonicotinamide (Fig. 5). Under these conditions, pyruvate does not stimulate microsomal *p*-nitroanisole *O*-demethylase activity directly (data not shown). Thus, it is concluded that pyruvate stimulates *p*-nitroanisole metabolism in hamster livers by increasing NADPH by pathways not involving the pentose phosphate shunt.

Both pyruvate dehydrogenase and pyruvate carboxylase may lead to increased NADPH generation from the metabolism of pyruvate (Fig. 6). The formation of acetyl CoA via pyruvate dehydrogenase could lead directly to increased tricarboxylate synthesis for isocitrate dehydrogenase-dependent synthesis of NADPH. Further, the availability of acetyl CoA could also increase citric acid cycle activity and increase levels of shuttle intermediates (e.g. malate, α-ketoglutarate; Table 3) required for the movement of reducing equivalents from the mitochondria into the cytosol (Fig. 6). Pyruvate carboxylase activity increases oxaloacetate which could also stimulate citric acid cycle activity. Alternatively, oxaloacetate could be reduced directly to malate, providing substrate for NADPH generation via cytosolic "malic" enzyme (Fig. 6). These possibilities cannot presently be differentiated since little is known of the regulation of pyruvate metabolism in hamster tissue. In rat liver, the K_m values of pyruvate dehydrogenase and pyruvate carboxylase for pyruvate are 0.02 and 0.4 mM respectively [23]. If similar enzyme affinities for pyruvate exist in hamster liver, it is possible that the pyruvate dehydrogenase pathway is involved, since the maximal effect of pyruvate was observed with only 0.1 mM (Fig. 3).

These experiments all suggest that mitochondrial metabolism is important in NADPH generation for mixed-function oxidation in perfused hamster livers. Furthermore, generation of NADPH from mitochondrial sources appears inadequate to maintain maximal rates of drug metabolism for extended periods.

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