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Rates of Pentose Cycle Flux in Perfused Rat Liver

Evaluation of the Role of Reducing Equivalents from the Pentose Cycle for Mixed-function Oxidation

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SUMMARY

Rates of NADPH production via the pentose phosphate cycle were determined in perfused livers from phenobarbital-treated rats by measuring $^{14}CO_2$ production from [1-^{14}C]glucose infused in the presence and absence of *p*-nitroanisole (0.2 mM), a substrate for mixed-function oxidation. In the fed state, basal rates of NADPH generation were 34–44 μmol/g/hr. *p*-Nitroanisole, which was metabolized at rates of 8.9 μmol/g/hr, stimulated pentose cycle-dependent NADPH production by 21–24 μmol/g/hr. Fasting for 24 hr prior to perfusion diminished pentose cycle flux by 80% and largely abolished the stimulation of the pentose cycle by *p*-nitroanisole. In contrast, rates of *p*-nitroanisole *O*-demethylation were only diminished slightly, to 5.7 μmol/g/hr. Fasting decreased hepatic glucose, glucose 6-phosphate, and 6-phosphogluconate contents drastically as expected. Pretreatment of rats with 6-aminonicotinamide, which is metabolized to a potent inhibitor of 6-phosphogluconate dehydrogenase, decreased rates of NADPH generation via the pentose cycle to 6.9 μmol/g/hr but did not alter rates of *p*-nitroanisole metabolism (8.8 μmol/g/hr). Basal rates of NADPH generation decreased from 38 to 26 μmol/g/hr during infusion of potassium cyanide (2 mM), an inhibitor of mitochondrial energy metabolism. Cyanide also decreased rates of *p*-nitroanisole *O*-demethylation by over 60%; however, stimulation of NADPH generation via the pentose cycle by *p*-nitroanisole was as great in the presence (17–21 μmol/g/hr) as in the absence of cyanide. Since rates of mixed-function oxidation were unaffected after virtually complete inhibition of the pentose cycle with 6-aminonicotinamide, it is concluded that reducing equivalents for the mixed-function oxidation of *p*-nitroanisole are not provided by the pentose cycle under these conditions.

INTRODUCTION

The mixed-function oxidase system has been studied primarily *in vitro* using isolated microsomal systems supplied with excess NADPH. However, in intact hepatocytes, several studies have demonstrated that NADPH can be rate limiting for mixed-function oxidation (1–3). For example, when *p*-nitroanisole is infused into livers from fed phenobarbital-treated rats, high rates of *p*-nitrophenol production are linear for less than 2 min and then decline to around 25% of the control value. Since oxygen and substrate were supplied in excess, it was concluded that the decrease in mixed-function oxidation was due to a decline in NADPH supply (1). However, Junge and Brand (4) proposed that the supply of NADPH exceeds that required for drug metabolism because rates of NADPH generation via the pentose cycle exceeded rates of mixed-function oxidation in hepatocytes supplied with glucose. Under these conditions, NADPH can be generated by the enzymes of the pentose cycle, glucose 6-phosphate (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.43). In the fed state, steady-state levels of cytosolic NADPH are either unchanged or increased during mixed-function oxidation (5, 6), consistent with the idea that rates of NADPH generation exceed rates of utilization via mixed-function oxidation. Although metabolism of glucose via the pentose pathway is thought to be a primary source of

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NADPH in livers from fed rats, it may also be derived from transhydrogenation of NADPH in mitochondria. In accord with this possibility, we found that treatment of rats with 6-aminonicotinamide, which is converted to an analog of $NADP^+$ that is a potent inhibitor of 6-phosphogluconate dehydrogenase (7, 8), did not inhibit *O*-demethylation of *p*-nitroanisole in the isolated perfused rat liver (9). Thus, either 6-aminonicotinamide treatment did not inhibit NADPH generation via the pentose cycle completely or alternate sources provided NADPH for mixed-function oxidation in livers of fed rats. Because little is known about rates of NADPH turnover in intact cells, experiments were designed to quantitate the contribution of the pentose cycle to the supply of reducing equivalents for mixed-function oxidation in livers from fed rats.

MATERIALS AND METHODS

Animals. Female Sprague-Dawley rats, 100–200 g, received sodium phenobarbital (1 mg/ml) in drinking water for 2 weeks prior to perfusion experiments to induce the mixed-function oxidase system (10). Fasted animals were deprived of food for 24 hr prior to use. Where indicated, rats were pretreated with 6-aminonicotinamide (70 mg/kg, i.p.) 6 hr before perfusion experiments.

Liver perfusion. Details of the perfusion technique have been described elsewhere (11). Livers were perfused at 37° with Krebs-Henseleit bicarbonate buffer, pH 7.4, saturated with an oxygen-carbon dioxide mixture (95:5) in a nonrecirculating system. *p*-Nitroanisole (0.2 mM) was dissolved in Krebs-Henseleit bicarbonate buffer, and the continuous formation of *p*-nitrophenolate ion was monitored spectrophotometrically as described previously (1). Potassium cyanide (2 mM), which was added to the perfusate in some experiments, had no effect on the pH of the perfusate or the spectral characteristics of *p*-nitrophenol. Rates of *p*-nitrophenolate production were calculated from the sum of free and conjugated *p*-nitrophenol produced by *O*-demethylation of *p*-nitroanisole. Sulfate and glucuronide conjugates of *p*-nitrophenol were determined by measuring *p*-nitrophenol released after incubation of 1.0 ml of perfusate with 0.5 ml of 180 mM phosphate buffer, pH 7.4, containing 250 units of purified β-glucuronidase and 25 units of sulfatase activity (Sigma), respectively, for 1.5 hr at room temperature. With this procedure, over 95% of all conjugates of *p*-nitrophenol were hydrolyzed.

Measurement of pentose phosphate cycle activity. Rates of NADPH generation from the pentose phosphate cycle were determined as described in detail previously (12). Briefly, samples of perfusate (20 ml) were taken during and after infusion of [1-^{14}C]glucose and were transferred into 500-ml Erlenmeyer flasks capped with silicon rubber stoppers. $^{14}CO_2$ was liberated by acidification of the perfusate and trapped in phenethylamine. Radioactivity was measured by scintillation spectrometry. When the infusion of [1-^{14}C]glucose was terminated, $^{14}CO_2$ production declined in a triphasic manner (Fig. 1). Phase I represents CO_2 production from the pentose phosphate cycle while phase II represents CO_2 production from the citrate cycle (12). The origin of the residual CO_2 in phase III remains unknown. The specific activity of intracellular glucose 6-phosphate was estimated from the specific activity of lactate isolated by anion exchange chromatography (12). Rates of pentose cycle activity were then calculated from the specific activity of glucose 6-phosphate and $^{14}CO_2$ produced from the pentose cycle (12). The rate of pentose cycle activity was doubled to yield the amount of NADPH formed since 2 mol of NADPH are generated per mol of glucose 6-phosphate metabolized to labeled CO_2 and ribulose 5-phosphate.

The activity of the pentose cycle was evaluated with two experimental designs. In Design I (Fig. 1), [1-^{14}C]glucose was infused into the liver for 20 min, and *p*-nitroanisole was added after 10 min of glucose infusion. Control experiments were performed in the absence of *p*-

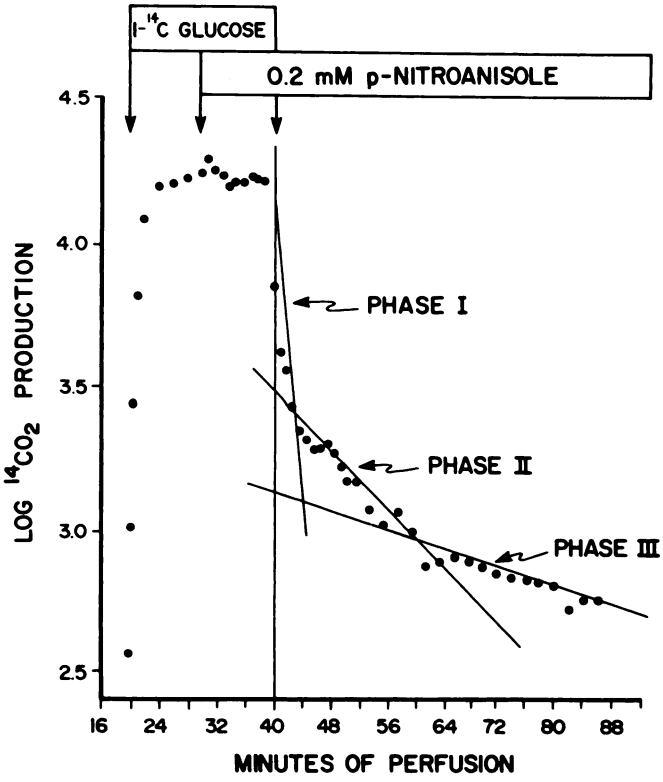

FIG. 1. *Kinetics of $^{14}CO_2$ washout following [1-^{14}C]glucose infusion*

[1-^{14}C]Glucose was infused into the perfusion fluid entering the liver at the rate of 2.5 × 10^6 cpm/min beginning 20 min after initiation of perfusion in livers from fed phenobarbital-treated rats. *p*-Nitroanisole (0.2 mM) was infused along with [1-^{14}C]glucose as designated by the horizontal bar and vertical arrows. Following termination of the glucose infusion (at 40 min), samples of perfusate were collected and analyzed for $^{14}CO_2$ as described under "Materials and Methods" and in more detail elsewhere (17).

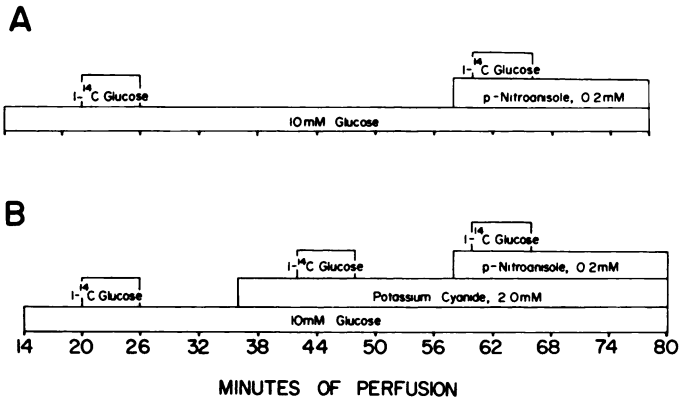

FIG. 2. *Experimental design employed to determine the effect of potassium cyanide on rates of p-nitrophenol production and generation of NADPH from the pentose cycle*

To accurately determine the effect of potassium cyanide on pentose cycle activity in the presence and absence of *p*-nitroanisole, several pulses of [1-^{14}C]glucose (2 × 10^6 cpm) were delivered allowing multiple measurements of pentose cycle activity to be made in the same liver. Conditions are described in Table 3.

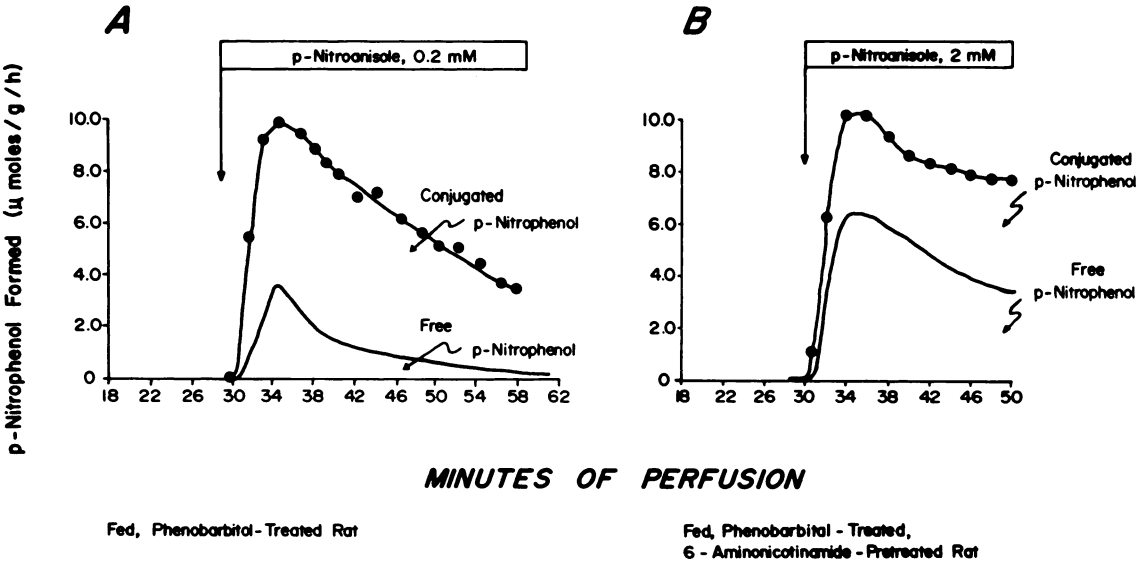

FIG. 3. *A.* Kinetics of *p*-nitrophenol production from the *O*-demethylation of *p*-nitroanisole. *p*-Nitroanisole (0.2 mM) was infused as designated by the horizontal bars and vertical arrows. Rates of free and conjugated *p*-nitrophenol production were calculated as described under "Materials and Methods." [1-^{14}C]Glucose was infused as described in the legend to Fig 1. B. Effect of 6-aminonicotinamide pretreatment on *p*-nitroanisole *O*-demethylation. Kinetics of *p*-nitrophenolate ion production in a liver from a fed phenobarbital-treated rat 6 hr after injection with 6-aminonicotinamide (70 mg/kg, i.p.). Other conditions were as described in the legend to Fig. 1.

nitroanisole. In Design 2 (Fig. 2), 6-min pulses of [1-^{14}C]glucose were infused before or during the infusion of *p*-nitroanisole and cyanide. Comparable values for NADPH production in the absence or presence of added glucose were obtained with both designs (see "Results").

Analytical measurements. Glucose, glucose 6-phosphate, and 6-phosphogluconate were determined by standard enzymatic procedures (13, 14) in $HClO_4$ extracts of livers which were freeze clamped with aluminum tongs chilled in liquid N_2.

p-Nitroanisole O-demethylation in liver homogenates. Livers from phenobarbital-treated rats were homogenized (0.3 g/ml) in Krebs-Henseleit bicarbonate buffer and centrifuged at 2500 × *g* for 10 min. Assays were performed in 25-ml Erlenmeyer flasks containing Krebs-Henseleit bicarbonate buffer, 0.5 mM *p*-nitroanisole, 1 ml of supernatant, and an NADPH-generating system consisting of 0.4 mM $NADP^+$, 30 mM isocitrate, and 0.2 unit of isocitrate dehydrogenase (Sigma), in a final volume of 2.0 ml. Incubations were initiated by the addition of the NADPH-generating system (0.2 ml) and were terminated after 10 min by the addition of 0.5 ml of $HClO_4$ (0.6 N). The mixture was centrifuged at 2500 × *g* for 3 min to remove precipitated protein, after which 200 μl of supernatant was added to 2.0 ml of 0.5 M Tris, pH 7.4. *p*-Nitrophenol concentration was determined spectrophotometrically (436 nm) employing an extinction coefficient of $\epsilon_{436} = 7.11\ mM^{-1}\ cm^{-1}$.

RESULTS

p-Nitroanisole O-demethylation. The kinetics of formation of *p*-nitrophenol from *p*-nitroanisole is depicted in Fig. 3A. Following the infusion of 0.2 mM *p*-nitroanisole, rates of *p*-nitrophenol formation reached maximal rates of near 9 μmol/g/h (Tables 1 and 3) in 6 min and declined by about 50% over 30 subsequent min of perfusion. Glucuronide and sulfate conjugates represented 70–80% of the product formed in livers from fed rats. Maximal rates of *p*-nitroanisole *O*-demethylation were about 6 μmol/g/h in livers from fasted rats (Table 1).

NADPH production from the pentose cycle. Basal rates of NADPH production from the pentose phosphate cycle were around 34 and 7 μmol/g/h in livers from fed and fasted phenobarbital-treated rats, respectively (Table 1). The addition of *p*-nitroanisole stimulated NADPH generation via the pentose cycle by about 23 μmol/g/h in livers from fed rats (Tables 1 and 3). In contrast, average rates of pentose cycle-dependent NADPH generation in livers from fasted rats were not altered by the addition of *p*-nitroanisole (Table 1).

Intermediates of the pentose cycle. Intermediates of the pentose cycle were measured in livers from fed and fasted phenobarbital-treated rats in the presence and absence of *p*-nitroanisole (Table 2). As expected, tissue levels of glucose, glucose 6-phosphate, and 6-phosphogluconate were diminished 80–90% by fasting (Table 2). In livers from fed but not fasted rats, glucose, glucose 6-phosphate, and 6-phosphogluconate tended to increase following the addition of *p*-nitroanisole (Table 2).

Effect of 6-aminonicotinamide and potassium cyanide on pentose cycle activity and p-nitroanisole O-demethylation. Rates of *p*-nitroanisole metabolism and NADPH generation were measured following pretreatment of rats with 6-aminonicotinamide, which is synthesized into an analog of $NADP^+$. This analog is a strong inhibitor of 6-phosphogluconate dehydrogenase (7) and should decrease flux of hexose in the pentose cycle. In livers from 6-aminonicotinamide-treated rats, basal rates of NADPH generation via the pentose cycle were nearly undetectable (<3 μmol/g/h) and were not altered by the addition of *p*-nitroanisole (Table 1). Under these conditions, rates of *p*-nitrophenol production (8.8 μmol/g/h) were identical to rates observed in livers of rats which had not been treated with 6-aminonicotinamide (8.9 μmol/g/h; Table 1).

In contrast, the addition of potassium cyanide (2 mM), an inhibitor of oxidative phosphorylation, decreased *p*-nitrophenol formation from *p*-nitroanisole to 2.7 μmol/g/h (63% inhibition) in livers from fed rats (Table 3) confirming similar experiments performed by Reinke *et*

TABLE 1

p-Nitrophenol production from p-nitroanisole and generation of NADPH from the pentose cycle in perfused livers from phenobarbital-treated rats

Values represent rates of *p*-nitrophenol production following cleavage of conjugates as described under "Materials and Methods." Rates of NADPH production were determined in the presence or absence of *p*-nitroanisole under conditions similar to those depicted in Fig. 1. Rats were treated with 6-aminonicotinamide (70 mg/kg, i.p.) 6 hr prior to perfusion experiments. Values are means ± SE taken from 4–18 livers/group.

Nutritional state	Addition	*p*-Nitrophenol production	NADPH production	Increase in NADPH due to *p*-nitroanisole
			μmol/g/h	
Fed	None		33.7 ± 3.8	
	p-Nitroanisole	8.9 ± 2.1	58.1 ± 8.7[a]	24.4
Fed + 6-aminonicotinamide	None		2.8 ± 0.9[b]	
	p-Nitroanisole	8.8 ± 0.9	5.8 ± 1.2	3.0
Fasted	None		6.9 ± 2.4[b]	
	p-Nitroanisole	5.7 ± 0.9[c]	9.3 ± 1.9	2.4
	p-Nitroanisole + potassium cyanide	1.2 ± 0.5[b]		

[a] $p < 0.01$ for the comparison with livers from fed untreated rats using Student's *t* test.

[b] $p < 0.001$ for the comparison with livers from fed untreated rats using Student's *t* test.

[c] $p < 0.05$ for the comparison with livers from fed untreated rats using Student's *t* test.

TABLE 2

Effect of nutritional state on cellular intermediates of the pentose cycle in perfused rat livers

Livers from phenobarbital-treated rats were clamped in aluminum tongs chilled in liquid nitrogen after 26 min of perfusion. *p*-Nitroanisole (0.2 mM) was infused after 20 min of perfusion. Metabolite concentrations were measured in freeze-clamped livers as described under "Materials and Methods." Values are means ± SE from 4–5 livers.

Nutritional state	Glucose	Glucose 6-phosphate	6-Phosphogluconate
	μmol/kg liver, wet weight		
Fed			
Control	430 ± 265	57 ± 26	5 ± 2
p-Nitroanisole	2195 ± 916	98 ± 24	11 ± 3
Fasted			
Control	27 ± 18	12 ± 6	0.8 ± 0.4
p-Nitroanisole	83 ± 22	15 ± 4	0.6 ± 0.3

al. (15). In the fasted state, rates of *p*-nitrophenol production were only 1.2 μmol/g/h in the presence of cyanide (Table 1). Concentrations of potassium cyanide used in the perfused liver have been reported to inhibit microsomal *p*-nitroanisole *O*-demethylation slightly (15). However, experiments using isolated microsomes do not take into account the potential binding sites for cyanide ion which exist in the intact cell. To attempt to address the question of the effect of cyanide on *p*-nitroanisole *O*-demethylation under conditions similar to those in the perfused liver, incubations were carried out with a 2500 × *g* supernatant of a 1:3 homogenate in the presence of an NADPH-generating system. Under these conditions, cyanide (2 mM) had no effect on *p*-nitroanisole *O*-demethylation (data not shown).

The effect of potassium cyanide on the pentose cycle was also investigated with the experimental design depicted in Fig. 2, A and B. In livers from fed phenobarbital-treated rats, basal rates of NADPH generation of around 44 μmol/g/h were increased by about 21 μmol/g/h upon the addition of *p*-nitroanisole (Table 3). In the presence of potassium cyanide, basal rates of NADPH generation via the pentose cycle were diminished from 38.5 to 26.0 μmol/g/h (Table 3), most likely due to inhibition of endogenous NADPH-utilizing systems (16–19). In the presence of cyanide, the addition of *p*-nitroanisole increased NADPH production by more than 17 μmol/g/h (Table 3). Thus, the increase in rates of NADPH production by the pentose cycle during infusion of *p*-nitroanisole was unaltered by cyanide (Table 3). Moreover, cyanide did not alter the increase in oxygen uptake observed following the addition of *p*-nitroanisole (Table 3).

DISCUSSION

NADPH turnover in the liver. In a study comparing hamsters, rats, and mice, the content of NADPH in the liver was found to be relatively constant, ranging from 112–282 μmol/kg, wet weight (20). The constancy of the intracellular pool of NADPH is surprising since it is utilized at high rates for monooxygenation, GSSG reduction, and fatty acid and cholesterol synthesis (20–23). In perfused livers from 3-methylcholanthrene-treated C57BL/6J mice, the addition of *p*-nitroanisole did not alter NADPH content, even though rates of *p*-nitrophenol formation via mixed-function oxidation were over 40 μmol/g/h (21). Without resynthesis, the NADPH pool in mouse liver would be depleted completely in less than 40 sec via monooxygenation alone under these conditions (19). Since NADPH concentration remains relatively constant during high rates of mixed-function oxidation, NADPH-generating systems such as the pentose cycle and the mitochondrial energy-linked transhydrogenase must be highly active in intact hepatocytes.

In spite of its central role in biosynthetic reactions, little is known about NADPH turnover in the liver. Many NADPH-synthesizing enzymes such as glucose 6-phosphate dehydrogenase are activated by $NADP^+$ and inhibited by NADPH (22). Thus, rates of NADPH synthesis are most likely regulated by rates of NADPH utilization. Since it is virtually impossible to measure the rates of all the metabolic pathways which synthesize and utilize NADPH, we have obtained information in this study under a variety of experimental conditions on one highly active NADPH synthetic pathway, the pentose cycle, and on one active NADPH-utilizing pathway, the mixed-function oxidase system. The latter requires 1 mol of NADPH to generate 1 mol of oxidized product. Comparisons of the stoichiometry between rates of NADPH synthesis by the pentose cycle and utilization by the mixed-function oxidase pathway in intact cells can be made; however, the data must be interpreted with caution, since other highly active NADPH synthetic and utilizing systems occur simultaneously and are most

TABLE 3

Effect of potassium cyanide on p-nitrophenol production and generation of NADPH from the pentose cycle in perfused livers from fed phenobarbital-treated rats

In order to determine accurately the effect of potassium cyanide on pentose cycle activity in the presence and absence of *p*-nitroanisole, several pulses of [1-^{14}C]glucose (2×10^6 cpm) were delivered so that multiple measurements of pentose cycle activity could be made in the same liver. Glucose (10 mM) was infused throughout the experiments to ensure adequate carbohydrate substrate throughout the experiment. Two basic experimental designs were employed (see Fig. 2). In experiment A, [1-^{14}C]glucose was added at 20 and 58 min of perfusion, and *p*-nitroanisole infusion was initiated at 56 min. In experiment B, potassium cyanide was infused beginning at 36 min, and [1-^{14}C]glucose was added at 20, 42, and 58 min. *p*-Nitroanisole infusion was initiated 2 min prior to the third pulse of [1-^{14}C]glucose. Following termination of each infusion of [1-^{14}C]glucose (6 min later) multiple samples of perfusate were collected, and $^{14}CO_2$ was determined as described under "Materials and Methods." Values are means ± SE taken from 4 livers/group. Statistics were performed using a matched paired *t* test.

Experiment	NADPH production				*p*-Nitrophenol production	Increase in oxygen uptake following *p*-nitroanisole
	Basal	+ Potassium cyanide	+ *p*-Nitroanisole	Change due to *p*-nitroanisole		
	μmol/g/h				*μmol/g/h*	
A	44.0 ± 10.0		65.5 ± 10.7[a]	21.5 ± 3.9	8.1 ± 0.2	13.5 ± 2.0
B	38.5 ± 4.4	26.0 ± 2.3	43.2 ± 2.6[b]	17.3 ± 2.1	2.7 ± 0.9[c]	14.3 ± 2.7

[a] $p < 0.01$ for the comparison with basal values.
[b] $p < 0.01$ for the comparison with potassium cyanide infusion in the absence of *p*-nitroanisole.
[c] $p < 0.01$ for the comparison with corresponding values in experiment A.

likely altered by nutritional state and addition of drugs and metabolic inhibitors.

Reducing equivalents for mixed-function oxidation in livers from fed and fasted rats. In perfused livers from fasted rats, rates of NADPH generation via the pentose cycle were only 6.8 μmol/g/h in the absence of added glucose (Table 1). In the fasted state, pentose cycle flux is most likely limited by substrate supply (23), because fasting decreases glucose, glucose 6-phosphate, and 6-phosphogluconate (Table 2). The increase in the rate of pentose cycle flux due to addition of *p*-nitroanisole (2.4 μmol/g/h) was less than the rate of *p*-nitrophenol production via mixed-function oxidation in the fasted state (5.7 μmol/g/h, Table 1). Thus, it appears likely that reducing equivalents for mixed-function oxidation must be supplied by a source other than the pentose cycle in the fasted state. The most likely source of these reducing equivalents is mitochondria since a number of inhibitors of mitochondrial function diminished mixed-function oxidation in livers from fasted rats. For example, 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation (24) and 2-bromooctanoate, an inhibitor of β-oxidation, diminished mixed-function oxidation by 60–70% in the fasted state (9, 25). Furthermore, ethanol (26), an inhibitor of the citric acid cycle, and antimycin A (3) and KCN ((15) Table 1), inhibitors of oxidative phosphorylation, also diminished mixed-function oxidation by 60–80%. NADH can supply reducing equivalents for *p*-nitroanisole *O*-demethylation *in vitro* (27, 28). However, because KCN increases NADH in the perfused liver yet inhibits monooxygenation, it seems unlikely that this occurs in intact cells.

In the fed state, reducing equivalents for mixed-function oxidation may arise via the oxidative enzymes of the pentose cycle as well as the mitochondria. In the presence of potassium cyanide, the *O*-demethylation of *p*-nitroanisole was decreased by 67% in perfused livers from fed rats (Fig. 3). This inhibition was not due to a direct effect of cyanide on cytochrome P-450, since cyanide had no effect on *p*-nitroanisole metabolism in liver homogenates. Thus, it is concluded that cyanide inhibits mixed-function oxidation predominantly by decreasing the supply of NADPH (15); however, from these data one cannot determine if the effect is on the pentose cycle or the mitochondrial energy-linked transhydrogenase.

The fact that rates of NADPH generation by the pentose cycle were increased to a similar extent (17–21 μmol/g/h) when *p*-nitroanisole was infused in the presence or absence of cyanide even though mixed-function oxidation of *p*-nitroanisole was considerably lower (63%) during cyanide infusion suggests that reducing equivalents for mixed-function oxidation are most likely not derived from the pentose cycle in the presence of cyanide. This hypothesis was tested with 6-aminonicotinamide, an inhibitor of the pentose cycle.

6-Aminonicotinamide is synthesized in the cell into 6-amino-NADP$^+$, an analog which is a potent inhibitor of 6-phosphogluconate dehydrogenase (7). Treatment with 6-aminonicotinamide results in a 700-fold increase in 6-phosphogluconate (8) and diminished rates of NADPH generation by the pentose cycle from nearly 60 to 5.8 μmol/g/h in the presence of *p*-nitroanisole (Table 1). In spite of this dramatic inhibition of the pentose cycle, the metabolism of *p*-nitroanisole to *p*-nitrophenol was unaffected (6-aminonicotinamide treated, 8.8 μmol/g/h; control, 8.9 μmol/g/h) (Fig. 3; Table 1). Thus, it is concluded that the mixed-function oxidation of *p*-nitroanisole must derive reducing equivalents from sources other than the pentose cycle in the presence of 6-aminonicotinamide.

The increase in rates of NADPH generation observed in the presence of *p*-nitroanisole and cyanide (Table 3) could stem from activation of an alternative NADPH-utilizing process by *p*-nitroanisole. For example, NADPH turnover could increase as a result of greater hydrogen peroxide production from uncoupling of the microsomal cytochrome P-450 system in the presence than in the absence of cyanide. Hydrogen peroxide formed from mixed-function oxidation could be decomposed by glutathione peroxidase (29), and oxidized glutathione re-

duced at the expense of NADPH (19). This hypothesis is supported by the fact that oxygen uptake, which is required for hydrogen peroxide formation, was increased to a similar extent by *p*-nitroanisole in the presence and absence of cyanide (Table 3).

Since rates of NADPH synthesis via the pentose cycle were 3 times greater than rates of mixed-function oxidation in the fed state (Table 1), it may seem reasonable to conclude, as did Junge and Brand (4), that the pentose cycle can provide all of the reducing equivalents for monooxygenation in the fed state. However, this interpretation focuses on only one NADPH-synthesizing pathway (pentose cycle) and one NADPH-utilizing pathway (mixed-function oxidation). A large proportion of NADPH is utilized for pathways other than mixed-function oxidation (e.g., fatty acid synthesis (30), the reduction of oxidized glutathione (31), flavoprotein-dependent monooxygenation (32), etc.). However, by using inhibitors of the pentose cycle (6-aminonicotinamide) and mitochondrial oxidations (cyanide) and measuring rates of pentose cycle and mixed-function oxidation, data from this study have shown that mitochondrial oxidations supply reducing equivalents for the mixed-function oxidation of *p*-nitroanisole in the fed state. These experiments are consistent with previous work which indicated that 50–70% of the NADPH utilized for the metabolism of 7-ethoxycoumarin to 7-hydroxycoumarin was supplied via mitochondrial oxidations (33).

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