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Alterations in Nicotinamide and Adenine Nucleotide Systems during Mixed-Function Oxidation of *p*-Nitroanisole in Perfused Livers from Normal and Phenobarbital-Treated Rats

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The contents of adenine nucleotides as well as steady-state concentrations of a number of glycolytic, pentose phosphate-pathway and tricarboxylic acid-cycle intermediates were measured in extracts of livers from normal and phenobarbital-treated rats that were perfused with *p*-nitroanisole. Metabolites were measured in livers that were freeze-clamped during periods of maximal rates of drug metabolism. Treatment of rats with phenobarbital increased rates of *p*-nitroanisole *O*-demethylation approx. fivefold. The concentrations of lactate, xylulose 5-phosphate and ribulose 5-phosphate were increased by phenobarbital treatment, whereas that of fructose 1,6-bisphosphate declined. Perfusion of livers with *p*-nitroanisole produced significant increases in 6-phosphogluconate and ribulose 5-phosphate in livers from phenobarbital-treated rats, but not in livers from control rats. Treatment of rats with phenobarbital caused [$NADP^+$]/[NADPH] to change in the direction of more oxidation, as calculated from measured concentrations of 6-phosphogluconate and ribulose 5-phosphate; however, the [$NADP^+$]/[NADPH] ratio calculated from 'malic' enzyme was not changed. Additions of *p*-nitroanisole produced a reduction of $NADP^+$ as calculated from 6-phosphogluconate dehydrogenase activity, but did not alter the [$NADP^+$]/[NADPH] ratio calculated from substrates assumed to be in equilibrium with 'malic' enzyme. Activities of both glucose 6-phosphate dehydrogenase and 'malic' enzyme were increased by phenobarbital treatment. NAD^+ became more reduced as a result of phenobarbital treatment; however, perfusion of livers with *p*-nitroanisole did not cause a change in the oxidation–reduction state of this nucleotide. Concentrations of adenine nucleotides in livers were not altered significantly by treatment of rats with phenobarbital; however, a significant decline in the [ATP]/[ADP] ratio occurred during mixed-function oxidation of *p*-nitroanisole in livers from phenobarbital-treated rats, but not in livers from normal rats. Perfusion of livers with two other substrates for mixed-function oxidation, hexobarbital and aminopyrine, produced an increase in the [$NADP^+$]/[NADPH] ratio calculated from 'malic' enzyme. In contrast with livers perfused with *p*-nitroanisole, there was no significant change in adenine nucleotides in livers exposed to hexobarbital or aminopyrine. Addition of 2,4-dinitrophenol (25 μM) to the perfusate containing aminopyrine decreased the [ATP]/[ADP] ratio and tended to prevent the oxidation of NADPH observed with aminopyrine alone. Thus in the presence of an uncoupler of oxidative phosphorylation, NADPH generation may exceed its utilization via mixed-function oxidation.

The metabolism of endogenous and foreign compounds by hepatic mixed-function oxidation is influenced by a wide array of biochemical substrates, including fats (Norred & Wade, 1972), proteins (Kato *et al.*, 1968; McLean & McLean, 1966) and carbohydrates (Peters & Strother, 1972). Mixed-function oxidation may be altered by these substrates through changes in intracellular nicotinamide and adenine nucleotide systems, since this process requires NADPH, and high-energy phosphate is utilized for subsequent conjugation reactions (Cour *et al.*, 1975). Studies of the inhibitory action of certain intermediates of the tricarboxylic acid cycle on oxidative *N*-dealkylation reactions indicate that changes of the mitochondrial oxidation–reduction state may be associated with altered rates of

metabolism of drugs by the microsomal mixed-function oxidase system (Moldeus *et al.*, 1973). Further, there is evidence that ultrastructural contacts occur between the endoplasmic reticulum and mitochondria (Denk *et al.*, 1976).

By using haemoglobin-free perfused livers from phenobarbital-treated rats that were deprived of food, we observed that the rate of *p*-nitroanisole *O*-methylation is enhanced by including glucose or gluconeogenic precursors in the perfusion medium (Thurman *et al.*, 1977). These findings raise the possibility that generation of reduced cofactor required for high rates of mixed-function oxidation in phenobarbital-treated rats that are deprived of food is influenced by available stores of carbohydrate. Further, rates of *p*-nitroanisole *O*-demethylation remain constant for only a short time (1–2 min) in livers from fed phenobarbital-treated rats (Thurman *et al.*, 1977). This rapid decline in the rate of mixed-function oxidation cannot be explained by smaller amounts of drug, substrate or oxygen supply. Consequently, it has been suggested that during high rates of mixed-function oxidation, NADPH also becomes rate-limiting in the fed state (Thurman *et al.*, 1977).

In the present study, interactions between intermediary metabolism and mixed-function oxidation were determined by examining changes in the steady-state concentrations of selected intermediates of the glycolytic, pentose phosphate and oxidative tricarboxylic acid pathways in livers from normal and phenobarbital-treated rats that were perfused in the presence and absence of *p*-nitroanisole, a model substrate for mixed-function oxidation. The oxidation–reduction state of the cytoplasmic NAD and NADP systems were calculated from measured substrates and equilibrium constants for lactate dehydrogenase (EC 1.1.1.27), 6-phosphogluconate dehydrogenase (EC 1.1.1.44) and 'malic' enzyme (EC 1.1.1.38) (Krebs & Veech, 1969). The data indicate that phenobarbital treatment alters the oxidation–reduction state of both the NADP and NAD systems, and increases the activities of several $NADP^+$-dependent dehydrogenases. Moreover, *O*-demethylation of *p*-nitroanisole is accompanied by a significant increase in the tissue content of 6-phosphogluconate, a key intermediate in the pentose phosphate pathway.

Methods

Animals

Livers were obtained from female Sprague–Dawley rats (120–140 g). Hepatic microsomal mixed-function oxidation was induced in some animals by administering phenobarbital (1 mg/ml in drinking water) 2 weeks before perfusion experiments (Marshall & McLean, 1969).

Liver perfusion and continuous determination of p-nitrophenol formation from p-nitroanisole

Livers were perfused with Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932), pH 7.4, by procedures described previously (Scholz *et al.*, 1973; Thurman *et al.*, 1977). Perfusions were carried out at 37°C with a haemoglobin-free perfusion medium to allow the A_{436} of *p*-nitrophenol to be continuously monitored. All livers were perfused with Krebs–Henseleit bicarbonate buffer for 20 min before perfusion with solutions containing *p*-nitroanisole. Preliminary experiments indicated that *p*-nitrophenol could not be determined in the presence of haemoglobin. The effluent perfusate was collected via a cannula placed in the vena cava, and was divided to direct a portion to a flow cuvette placed in a recording Eppendorf spectrophotometer and another portion to an oxygen electrode. Portions of the perfusate were also sampled for metabolite measurements. Concentrations of *p*-nitrophenolate in the effluent were determined by using the millimolar absorption coefficient (ε_{436} = 5.19 litre·mmol^{-1}·cm^{-1} at pH 7.4) as described previously (Thurman *et al.*, 1977). Total rates of *p*-nitrophenol production were calculated from the concentration in the effluent perfusate, the flow rate and the liver weight.

Metabolite measurement

Metabolites were determined in $HClO_4$ extracts of livers that had been freeze-clamped with tongs chilled in liquid N_2 (Wollenberger *et al.*, 1960). Samples of frozen liver weighing about 200 mg were powdered, and were extracted with 0.3 M-$HClO_4$ as described previously (Kauffman *et al.*, 1969). The protein-free acid extracts were neutralized with 2 M-$KHCO_3$ and stored at −80°C until assayed for metabolites.

All intermediates of the Embden–Meyerhof pathway, and glutamate, were measured enzymically by fluorimetric procedures described previously (Lowry & Passonneau, 1972). Intermediates of the tricarboxylic acid cycle were measured by methods described by Goldberg *et al.* (1966) and those of the pentose phosphate pathway by procedures described by Kauffman *et al.* (1969).

Determination of enzyme activities

Enzyme activities were determined in samples of fresh livers that were homogenized in 10 vol. of 0.25 M-sucrose/2 mM-$CaCl_2$/5 mM-sodium phosphate, pH 7.0. All enzyme assays were performed at room temperature (23–25°C), and are based on the direct fluorimetric determinations of the initial rates of oxidation or reduction of NADP after addition of appropriate substances. '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) were determined as described previously (Luine & Kauffman, 1971). Activities of the above enzymes were linear with respect to time for at least 10min. Glutathione reductase (EC 1.6.4.2) was determined fluorimetrically by an adaptation of the method described by Racker (1955), in which the concentration of NADPH was lowered to 10μM to avoid fluorescence quenching. The oxidation of NADPH in the presence of oxidized glutathione was linear for 2min.

Calculations

Calculations of the ratios of free oxidized and reduced nicotinamide nucleotides were made from the expression:

$$\frac{\text{Free [NAD(P)}^+\text{]}}{\text{Free [NAD(P)H]}} = \frac{1}{K} \times \frac{\text{[oxidized substrate]}}{\text{[reduced substrate]}}$$

where K represents the thermodynamic equilibrium constant of the reaction (Williamson *et al.*, 1967). These calculations rest on the assumption that the oxidized and reduced substrates of selected nicotinamide nucleotide-linked dehydrogenases are in equilibrium with the free nucleotides. The cytoplasmic [NAD^+]/[NADH] ratios were calculated by using the equilibrium constant of 0.111mM for lactate dehydrogenase (Williamson *et al.*, 1967). Cytoplasmic [$NADP^+$]/[NADPH] ratios were calculated from the equilibrium constant of 34.4mM for 'malic' enzyme (Krebs & Veech, 1969) and 0.170M for 6-phosphogluconate dehydrogenase (Villet & Dalziel, 1969). The calculations also assume an intracellular pH of 7.0 and a CO_2 concentration of 1.16mM (Krebs & Veech, 1969). Statistical evaluation of values obtained in separate groups of livers were made by using Student's t test.

Materials

All enzymes except lactate dehydrogenase and glycerophosphate dehydrogenase (EC 1.1.1.94) were purchased from Boehringer, Mannheim, Germany. Heart lactate dehydrogenase was purchased from Worthington, Freehold, NJ, U.S.A. Phosphorylated substrates, nicotinamide nucleotides and glycerophosphate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were reagent grade from standard sources (Merck, Darmstadt, Germany; Fisher Chemical Co., Silver Springs, MD, U.S.A.).

Results

Metabolism of p-nitroanisole in perfused livers

Typical records of *p*-nitroanisole *O*-methylation in livers from normal and phenobarbital-treated rats are shown in Fig. 1. The maximal rate of drug metabolism in livers from phenobarbital-treated rats was approx. fourfold that observed in control livers. The secondary decline in phenobarbital-treated rats occurred consistently 2–3min after exposing the livers to *p*-nitroanisole (Fig. 1). This decline was never observed in livers from normal rats. In all

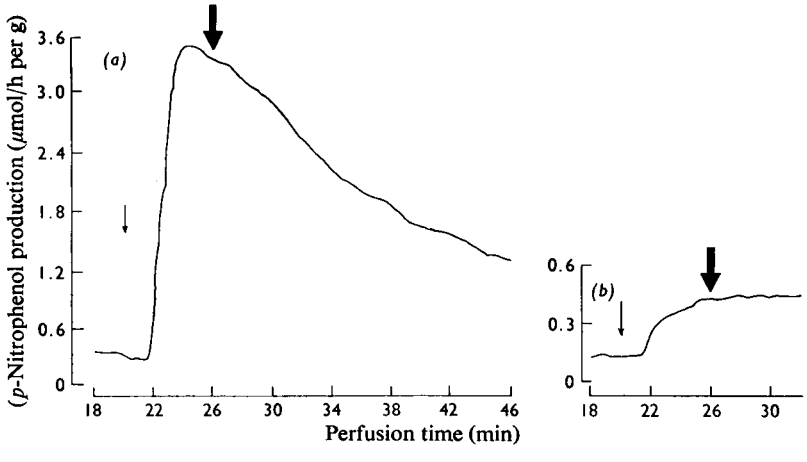

Fig. 1. *p-Nitrophenol production by livers from phenobarbital-treated (a) and normal (b) rats*
Livers were perfused and the absorbance of *p*-nitrophenol was determined in the effluent perfusate as described in the Methods section. The small arrows indicate the time when *p*-nitroanisole (0.2mM) infusions were started, and the large wide arrows indicate times at which livers were freeze-clamped.

Table 1. *Metabolite concentrations in perfused livers from normal and phenobarbital-treated rats*

Values are means ± S.E.M. for livers from six normal rats and seven phenobarbital-treated rats. Livers were perfused with Krebs–Henseleit buffer alone and freeze-clamped at the time intervals shown in Fig. 1. $*P<0.05$, normal versus phenobarbital-treated; $**P<0.01$.

	Concn. (μmol/kg wet wt.)	
	Normal	Phenobarbital-treated
Glucose 6-phosphate	40.9 ± 2.5	40.9 ± 10.6
Fructose 1,6-bisphosphate	28.6 ± 3.1	11.6 ± 2.1*
Dihydroxyacetone phosphate	39.1 ± 10.1	38.4 ± 4.6
Pyruvate	70.2 ± 6.6	93.2 ± 12.8
Lactate	227.8 ± 49.3	638.1 ± 138.3**
6-Phosphogluconate	7.3 ± 2.8	6.7 ± 1.4
Ribulose 5-phosphate	7.4 ± 2.4	39.3 ± 4.3**
Xylulose 5-phosphate	8.6 ± 1.9	35.7 ± 3.4**
Malate	78.4 ± 14.2	68.8 ± 8.5
α-Oxoglutarate	86.5 ± 21.5	60.9 ± 18.0
Glutamate	863.4 ± 105.7	699.5 ± 77.5

experiments, livers were freeze-clamped at 6 min after the addition of drug to the perfusate, because rates of *p*-nitrophenol production were maximal at this time. Control livers were perfused with Krebs–Henseleit buffer alone, and were freeze-clamped at the same time as the livers were perfused with drug.

Metabolite concentrations in livers from normal and phenobarbital-treated rats

Amounts of metabolites in perfused livers from normal and phenobarbital-treated rats in the absence of *p*-nitrophenol are compared in Table 1. Livers from rats treated with phenobarbital for 2 weeks contained significantly less fructose 1,6-bisphosphate than did livers from normal rats. Significant increases in lactate, xylulose 5-phosphate and ribulose 5-phosphate were also noted in these tissues. Concentrations of xylulose 5-phosphate and ribulose 5-phosphate were more than 4 times as high in livers from phenobarbital-treated rats as in livers from normal rats. On the other hand, malate, α-oxoglutarate and glutamate concentrations tended to be lower in livers from fed phenobarbital-treated rats than those in normal livers, but the changes were not statistically significant. Concentrations of malate, pyruvate and glutamate noted above are comparable with values reported previously for perfused livers under conditions similar to those used in the present studies (Williamson *et al.*, 1969; Sies & Kandel, 1970). Phenobarbital treatment was without effect on glucose 6-phosphate, 6-phosphogluconate and dihydroxyacetone phosphate.

Table 2. *Effect of p-nitroanisole on metabolites in perfused livers from normal and phenobarbital-treated rats*

Livers were perfused with 0.2 mM-*p*-nitroanisole and freeze-clamped at the time invervals indicated in Fig. 1. Values are expressed as percentages of control values given in Table 1 and are means ± S.E.M. for five livers from normal rats and seven livers from phenobarbital-treated rats. Concentrations of metabolites that differ significantly from the respective values in Table 1 are indicated with asterisks: $*P<0.05$; $**P<0.01$.

	Content (% of control)	
Metabolite	Normal	Phenobarbital-treated
Glucose 6-phosphate	110 ± 8	208 ± 59
Fructose 1,6-bisphosphate	53 ± 11*	208 ± 29*
Dihydroxyacetone phosphate	117 ± 19	143 ± 12*
Pyruvate	117 ± 10	140 ± 24
Lactate	112 ± 18	271 ± 60*
6-Phosphogluconate	139 ± 27	536 ± 136**
Ribulose 5-phosphate	150 ± 19	143 ± 7*
Xylulose 5-phosphate	206 ± 43	124 ± 16
Malate	89 ± 7	103 ± 12
α-Oxoglutarate	129 ± 15	92 ± 17
Glutamate	113 ± 14	90 ± 11

Effect of p-nitroanisole infusion on metabolite concentrations

Perfusion of livers with *p*-nitroanisole produced qualitatively different alterations in concentrations of hepatic metabolites in tissues from normal and phenobarbital-treated rats (Table 2). A significant decrease in fructose 1,6-bisphosphate occurred in normal livers 6 min after infusion of the drug; however, amounts of other metabolites were not significantly altered. In contrast, fructose 1,6-bisphosphate, lactate and dihydroxyacetone phosphate increased significantly in livers from phenobarbital-treated rats exposed to *p*-nitroanisole. In addition, a very large increase in 6-phosphogluconate was observed in these livers (Table 2). The increase in lactate produced in livers of phenobarbital-treated rats by *p*-nitroanisole perfusion confirms previous observations that lactate production is stimulated by *p*-nitroanisole (Thurman *et al.*, 1977).

Ribulose 5-phosphate, the immediate product of the 6-phosphogluconate dehydrogenase reaction, was also increased in livers of phenobarbital-treated rats; however, xylulose 5-phosphate did not change. Concentrations of both of these intermediates were elevated in livers from normal rats, but these changes were not statistically significant. Xylulose 5-phosphate and ribulose 5-phosphate were present at equal concentrations in livers from both groups of animals, in contrast with the brain, where they exist in a molar ratio of approx. 3:1, as expected from equilibrium considerations (Kauffman *et al.*, 1969).

Table 3. *Adenine nucleotides in perfused livers from normal and phenobarbital-treated rats*

Adenine nucleotides were measured in extracts of livers described in Table 1. Values are means±S.E.M. for the numbers of animals shown in parentheses. Statistical comparisons refer to differences between control and *p*-nitroanisole-treated groups in each type of liver: $*P<0.05$.

	Concn. (μmol/kg wet wt.)			
	Normal		Phenobarbital-treated	
	Control (6)	*p*-Nitroanisole-treated (5)	Control (6)	*p*-Nitroanisole-treated (7)
ATP	1972±197	2404±212	1461±220	1349±210
ADP	525±59	584±71	231±48	552±105*
AMP	148±56	97±18	344±90	721±176
Total adenosine phosphates	2645±132	3085±261	2198±177	2530±40
[ATP]/[ADP]	4.1±0.6	4.3±0.5	6.6±1.6	2.2±0.6

Drug metabolism in phenobarbital-treated rats did not alter the concentrations of malate, α-oxoglutarate, glutamate, glucose 6-phosphate and pyruvate. Thus the major changes in intermediates associated with the generation of reducing equivalents appear to be restricted to cytoplasmic components. Glutamate, α-oxoglutarate, malate and pyruvate, which serve as substrates for various hydrogen shuttles involved in the transport of reducing equivalents across mitochondrial membranes (Borst, 1963; Williamson *et al.*, 1969), were not altered by phenobarbital treatment, nor did steady-state concentrations of these intermediates change in livers perfused with *p*-nitroanisole. In the absence of large amounts of fatty acids in the livers of well-fed rats, generation of reducing equivalents via the oxidative enzymes of the pentose phosphate pathway could readily serve as the major source of hydrogen for mixed-function oxidation.

Adenine nucleotides in livers from normal and phenobarbital-treated rats

Control livers from normal rats contained AMP, ADP and ATP in the approximate molar proportions of 1:4:13 (Table 3). During perfusion with *p*-nitroanisole, the [ATP]/[ADP] ratio in normal livers remained constant. However, phenobarbital treatment caused a significant decrease in concentrations of ADP, as reflected in a higher [ATP]/[ADP] ratio (Table 3). In contrast with normal livers, perfusion of livers from phenobarbital-treated rats with *p*-nitroanisole produced a significant decrease in the [ATP]/[ADP] ratio. Adenine nucleotide concentrations and ratios in livers from phenobarbital-treated rats were qualitatively different from control livers. Calculated adenylate kinase mass-action ratios $[ATP\times([AMP]/[ADP]^2)]$, which were approx. 1.1 in livers from normal rats, increased to 9.4 in livers from phenobarbital-treated rats. Perfusion of livers with *p*-nitroanisole produced approximately a twofold decrease in the adenylate kinase mass-action ratio in normal tissue and about a threefold decrease in livers from the phenobarbital-treated rats (Table 3).

Nicotinamide nucleotide oxidation–reduction states in livers from normal and phenobarbital-treated rats

The cytoplasmic oxidation–reduction state of NADP calculated from [malate]/[pyruvate] ratios and the equilibrium constant for 'malic' enzyme were essentially the same in livers from normal and phenobarbital-treated rats (Table 4). The calculated value of 0.0356 in these experiments is three times the value reported by Krebs & Veech (1969) for well-fed normal rats. Perfusion of livers with *p*-nitroanisole failed to produce significant changes in the $[NADP^+]$/[NADPH] ratio calculated from substrates assumed to be near equilibrium with 'malic' enzyme in either group.

In livers from untreated animals, the ratio $[NADP^+]$/[NADPH] calculated from measured values of ribulose 5-phosphate and 6-phosphogluconate was significantly lower than that calculated from substrates assumed to be in equilibrium with 'malic' enzyme (0.0113 versus 0.0356). The calculated value from 6-phosphogluconate dehydrogenase in normal livers is in good agreement with values of 0.0118 and 0.0108 calculated from measured substrates for 'malic' enzyme and isocitrate dehydrogenase in normal rats in a previous study (Krebs & Veech, 1969). In contrast with results obtained from measurements of malate and pyruvate, phenobarbital treatment resulted in a more oxidized NADP system as calculated from substrates in near-equilibrium with 6-phosphogluconate dehydrogenase, i.e. the $[NADP^+]$/[NADPH] ratio increased from 0.0113 to 0.0413. The latter ratio decreased in phenobarbital-treated livers on addition of *p*-nitroanisole, to 0.0222. This reduction of the cytoplasmic $NADP^+$–NADPH redox system in

Table 4. *Calculated [NADP⁺]/[NADPH] ratios in perfused livers from normal and phenobarbital-treated rats*
The values were calculated from data summarized in Table 1 and are expressed as means±S.E.M. for the number of animals shown in parentheses. *$P<0.05$ for difference from normal rat.

	[NADP⁺]/[NADPH] ratio calculated from:			
	'Malic' enzyme		6-Phosphogluconate dehydrogenase	
Addition	Normal	Phenobarbital-treated	Normal	Phenobarbital-treated
None	0.0356±0.0074 (6)	0.0411±0.0028 (6)	0.0113±0.0052 (4)	0.0413±0.0086* (6)
0.2mM-*p*-Nitroanisole	0.0397±0.0017 (5)	0.0669±0.0160 (6)	0.0127±0.0027 (5)	0.0222±0.0079 (7)

Table 5. *Calculated [NAD⁺]/[NADH] ratios in perfused livers from normal and phenobarbital-treated rats*
Values were calculated from the equilibrium constant for lactate dehydrogenase and measured values of lactate and pyruvate summarized in Table 1. *$P<0.05$; **$P<0.01$.

	[NAD⁺]/[NADH]	
Addition	Normal	Phenobarbital-treated
None	3103±550 (5)	1457±272* (6)
0.2mM-*p*-Nitroanisole	2497±183 (5)	1398±271** (6)

the presence of a substrate for mixed-function oxidation was unexpected, and is opposite to the oxidation produced by hexobarbital, another substrate for mixed-function oxidation (Sies & Kandel, 1970).

Treatment of rats with phenobarbital caused approximately a twofold change in the ratio [NAD⁺]/[NADH] (Table 5); however, this change was in the direction of reduction. The tendency of the [NAD⁺]/[NADH] ratio to change in the direction of reduction in the phenobarbital-treated rat, while the [NADP⁺]/[NADPH] ratio changed in the direction of oxidation, lends support to the hypothesis (Thurman & Scholz, 1970*a*) that the extramitochondrial pools of the two nucleotides are not in rapid equilibrium. The finding that perfusion of livers with *p*-nitroanisole altered the calculated [NADP⁺]/[NADPH] but not the [NAD⁺]/[NADH] ratio (Table 5) is also in accord with this hypothesis.

Effect of mixed-function-oxidation substrates and dinitrophenol on hepatic adenine nucleotides

In view of the failure of *p*-nitroanisole to increase the [NADP⁺]/[NADPH] ratio, we examined the effect of several drug substrates and an uncoupling agent on both adenine nucleotides and calculated ratios of nicotinamide nucleotides.

Adenine nucleotide amounts and [ATP]/[ADP] ratios were compared in livers perfused with *p*-nitroanisole, hexobarbital, aminopyrine and dinitrophenol plus aminopyrine (Table 6). In the presence of *p*-nitroanisole, ATP concentrations tended to decline, whereas ADP and AMP increased. These changes are reflected in a significant decline in the [ATP]/[ADP] ratio similar to that observed in the other experimental series (Table 3). In contrast, aminopyrine and hexobarbital failed to produce statistically significant changes in adenine nucleotide concentration or the [ATP]/[ADP] ratio. When aminopyrine was added in the presence of a known uncoupler of oxidative phosphorylation, dinitrophenol, significant increases in ADP and AMP were observed. Under these conditions, the [ATP]/[ADP] ratio was similar to that observed in the presence of *p*-nitroanisole (Table 6). The reason for the discrepancy in the values for AMP and the [ATP]/[ADP] ratios in the control values for phenobarbital-treated rats in the separate experimental series reported in Tables 3 and 6 is not clear. The two sets of experiments were carried out approximately 8 months apart, and the difference may reflect seasonal variations.

Effect of mixed-function-oxidation substrates and dinitrophenol on calculated nicotinamide nucleotide ratios

p-Nitroanisole and aminopyrine in the presence of 2,4-dinitrophenol failed to affect the calculated [NADP⁺]/[NADPH] ratio (Table 7). However, in accord with previous observations (Sies & Kandel, 1970), a tendency toward oxidation was observed with aminopyrine and hexobarbital ($0.05<P<0.1$). Although these drugs did not produce statistically significant changes in the [NAD⁺]/[NADH] ratios, the calculated ratios in the presence of aminopyrine, hexobarbital and aminopyrine plus dinitrophenol tended to be lower than control values (Table 7). Control values for the [NADP⁺]/[NADPH] ratios in Table 7 differ from those in Table 4. It is difficult to explain this difference; however, comparison within each set of experiments is justified, because each series was carried out over a relatively short period of time (7–10 days), and control and experimental livers were obtained randomly.

Table 6. *Adenine nucleotides in livers of phenobarbital-treated rats perfused with various drugs*

Values are averages ± S.E.M. obtained from three to five livers. Drugs were perfused at the following concentrations: *p*-nitroanisole, 0.2 mM; hexobarbital, 0.1 mM; aminopyrine, 0.5 mM; dinitrophenol, 25 μM. $*P<0.05$; $**P<0.01$, for difference from control.

	Contents (μmol/kg wet wt. of tissue)			
	ATP	ADP	AMP	[ATP]/[ADP]
Control (4)	1812 ± 66	484 ± 29	159 ± 28	3.77 ± 0.21
p-Nitroanisole (3)	1163 ± 417	772 ± 42**	245 ± 66	1.93 ± 0.69*
Hexobarbital (5)	1641 ± 98	508 ± 58	273 ± 77	3.40 ± 0.40
Aminopyrine (5)	1394 ± 192	506 ± 58	194 ± 73	2.91 ± 0.44
Dinitrophenol + aminopyrine (4)	1357 ± 207	814 ± 49**	386 ± 81*	1.73 ± 0.36**

Table 7. *Calculated ratios of oxidized and reduced nicotinamide nucleotides in livers of phenobarbital-treated rats perfused with various drugs*

Values were calculated from concentrations of pyruvate, lactate and malate in livers of phenobarbital-treated rats freeze-clamped at the time indicated in Fig. 1. The concentrations of each drug in the perfusate were as follows: *p*-nitroanisole, 9.2 mM; hexobarbital, 0.1 mM; aminopyrine, 0.5 mM; dinitrophenol, 25 μM. Values are averages ± S.E.M. for the numbers of livers given in parentheses.

	$\frac{[NAD^+]}{[NADH]}$ (Lactate dehydrogenase)	$10^2 \times \frac{[NADP^+]}{[NADPH]}$ ('Malic' enzyme)
Control (4)	1144 ± 287	1.96 ± 0.41
p-Nitroanisole (4)	806 ± 290	1.98 ± 0.43
Hexobarbital (5)	1164 ± 263	3.30 ± 0.51
Aminopyrine (5)	753 ± 199	2.75 ± 0.47
Dinitrophenol + aminopyrine (4)	621 ± 138	2.20 ± 0.56

Table 8. *Enzyme activities in homogenates of livers from normal and phenobarbital-treated rats*

Enzyme activities were measured in homogenates of whole livers. Values are averages ± S.E.M. from four livers. $*P<0.5$; $**P<0.01$, for difference from normal rats.

	Activity (μmol/min per g of protein)	
Enzyme	Normal	Phenobarbital-treated
Isocitrate dehydrogenase	64.2 ± 6.6	57.5 ± 5.2
Glutathione reductase	12.1 ± 1.4	14.2 ± 0.9
6-Phosphogluconate dehydrogenase	14.2 ± 0.9	14.6 ± 1.9
Glucose 6-phosphate dehydrogenase	4.42 ± 0.68	7.63 ± 0.80*
'Malic' enzyme	1.71 ± 0.14	2.50 ± 0.04**

NADPH-generating enzymes in livers from normal and phenobarbital-treated rats

To determine whether altered mixed-function oxidation of *p*-nitroanisole is accompanied by changes in the capacity of the liver to generate NADPH, we examined activities of the five major cytoplasmic NADPH-generating enzymes in normal and phenobarbital-treated livers (Table 8). Treatment of rats with phenobarbital produced significant increases in the activities of glucose 6-phosphate dehydrogenase and 'malic' enzyme. Thus induction of oxidative drug metabolism is accompanied by an enhanced capacity to generate NADPH via glucose 6-phosphate dehydrogenase and 'malic' enzyme. The activities of other NADPH-generating enzymes were not changed significantly by pretreatment with phenobarbital (Table 8).

Discussion

Effect of phenobarbital treatment on hepatic metabolites

The induction of mixed-function oxidation in rat liver by phenobarbital is accompanied by significant alterations in intermediates and enzyme activities associated with adenine and nicotinamide nucleotides. Consideration of these changes is important, since alterations in the capacity to generate reducing equivalents in the form of NADPH could well affect rates of mixed-function oxidation in the intact liver. On the basis of the above data, it is suggested that phenobarbital treatment increases metabolism via the pentose phosphate pathway in livers from well-fed rats. This suggestion is supported by the finding that pretreatment with phenobarbital elevated the activity of hepatic glucose 6-phosphate dehydrogenase (Table 8) and increased the concentrations of ribulose 5-phosphate and xylulose 5-phosphate (Table 1). Although changes in steady-state concen-

trations of metabolic intermediates do not necessarily reflect an increase in rates of metabolism, elevation of the two intermediates in the non-oxidative portion of the pentose phosphate pathway has been associated previously with increased flow of hexose through this metabolic route (Kauffman *et al.*, 1969). The observed increase in lactate in the presence of a decline in fructose 1,6-bisphosphate in livers of rats pretreated with phenobarbital is also consistent with the possibility that metabolism via the pentose phosphate pathway was increased by drug treatment.

The possibility that glycolysis was decreased in livers of phenobarbital-treated rats is suggested by the decrease in fructose 1,6-bisphosphate, which is known to occur in intact tissues when phosphofructokinase is inhibited (Lowry *et al.*, 1964). Elevation of hepatic [ATP]/[ADP] ratios (Table 3) in phenobarbital-treated rats leading to inhibition of this enzyme is most likely to be responsible for the observed decline in fructose 1,6-bisphosphate, since ATP is a strong inhibitor of phosphofructokinase (Lowry & Passonneau, 1966).

The increase in the [$NADP^+$]/[NADPH] ratio calculated from measured concentrations of 6-phosphogluconate and ribulose 5-phosphate in livers of phenobarbital-treated rats (Table 4) was unexpected in view of the increased activities of two NADPH-generating enzymes produced by the drug. Mechanisms contributing to the oxidized state of NADP in livers of phenobarbital-treated rats are not known; however, this change could contribute to enhanced carbon flux over the pentose phosphate pathway. It has been shown that the absolute amounts of $NADP^+$ and NADPH as well as the ratio of these two nucleotides are crucial in regulating glucose 6-phosphate dehydrogenase (Afolayan, 1972; Eggleston & Krebs, 1974; Procsal & Holten, 1972). In liver of normal, well-fed rats it has been estimated that the activities of the two oxidative enzymes of the pentose phosphate pathway are inhibited more than 98%, owing to the low [$NADP^+$]/[NADPH] ratio (Holten *et al.*, 1976). Under normal physiological conditions, it has been estimated that an increase in the [$NADP^+$]/[NADPH] ratio from 0.005 to 0.02 would produce approximately a sixfold increase in the activities of both glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Thus the high [$NADP^+$]/[NADPH] ratio observed in liver of phenobarbital-treated rats, coupled with the elevated amounts of glucose 6-phosphate dehydrogenase, could greatly enhance the metabolism of hexose via the pentose phosphate pathway in this tissue. In the absence of large amounts of fatty acids in the livers of well fed-rats, generation of reducing equivalents via the oxidative enzymes of the pentose pathway could readily serve as the major source of hydrogen for mixed-function oxidation.

Metabolic alterations accompanying mixed-function oxidation of p-nitroanisole

The possibility that *O*-demethylation of *p*-nitroanisole in the perfused liver is associated with altered metabolism via the pentose phosphate pathway is suggested by the large increase in 6-phosphogluconate and the somewhat smaller increase in ribulose 5-phosphate that occurred after introduction of the drug into the perfusate (Table 2). The increase in these two intermediates is most likely to be associated with enhanced metabolism via the pentose phosphate pathway. In a study of mixed-function oxidation of hexobarbital in isolated hepatocytes, Junge & Brand (1975) found that metabolism of this drug was accompanied by increased incorporation of [1-^{14}C]-glucose into CO_2 and C-1 of hexose 6-phosphate, suggesting increased metabolism via the pentose phosphate pathway. Further, these authors estimated that the generation of NADPH via the pentose phosphate pathway exceeded the rate of hexobarbital metabolism by a factor of 6 in normal rat liver and by a factor of 9.5 in livers of phenobarbital-treated rats.

Mixed-function oxidation of *p*-nitroanisole in livers of phenobarbital-treated rats may also be accompanied by enhanced glycolysis. Amounts of lactate in the liver (Table 2) as well as in the effluent perfusate (Thurman *et al.*, 1977) were increased after introduction of the drug to the liver. This increase in lactate was accompanied by significant increase in fructose 1,6-bisphosphate and dihydroxyacetone phosphate. Thus changes in glycolytic rate accompanying *O*-demethylation of *p*-nitroanisole may be mediated at the level of phosphofructokinase. Activation of phosphofructokinase could readily occur as a result of the decline in the [ATP]/[ADP] ratio in livers from phenobarbital-treated rats perfused with the drug (Table 3). This mechanism apparently is not operative in livers of normal rats, since the [ATP]/[ADP] ratio and the concentration of fructose 1,6-bisphosphate did not change during perfusion with *p*-nitroanisole. The increase in ADP during *p*-nitroanisole *O*-demethylation in livers of phenobarbital-treated rats is most probably due to uncoupling of oxidative phosphorylation by the relatively large amounts of *p*-nitrophenol which were generated as a consequence of mixed-function oxidation (Thurman *et al.*, 1977).

Earlier studies of the metabolism of hexobarbital in perfused livers of phenobarbital-treated rats indicated that mixed-function oxidation of this drug was associated with an oxidation of NADP (Sies & Kandel, 1970). The oxidation of nicotinamide nucleotides has also been detected by measurements of surface fluorescence in livers perfused with aminopyrine, another substrate for mixed-function oxidase, in the presence of gluconeogenic precursors (Thurman & Scholz, 1970*b*). In the present study, we failed to detect a significant change in the

[$NADP^+$]/[NADPH] ratio calculated from substrates presumed to be in equilibrium with 'malic' enzyme during *O*-demethylation of *p*-nitroanisole (Table 4). In addition, perfusion of livers from phenobarbital-treated rats with *p*-nitroanisole produced an unexpected decrease in the [$NADP^+$]/[NADPH] ratios calculated from measured values for 6-phosphogluconate and ribulose 5-phosphate. This decrease did not occur in livers from normal rats, in which rates of *p*-nitrophenolate production were very low (Fig. 1).

The failure of the [$NADP^+$]/[NADPH] ratios calculated from 'malic' enzyme and 6-phosphogluconate dehydrogenase to agree is difficult to reconcile with the hypothesis that substrates for these enzymes are in near-equilibrium with cytoplasmic pools of NADP. The disparity between calculated ratios may be related to compartmentation of the two activities within the liver or to the relatively low activity of 'malic' enzyme. Even though phenobarbital treatment significantly elevated the activity of 'malic' enzyme (Table 8), this activity remained the lowest of the five $NADP^+$-dependent activities measured.

Mechanism of enhanced NADPH generation in the presence of p-nitroanisole

The results presented in Table 7 verify that hexobarbital and aminopyrine tend to oxidize cytosolic NADP; however, oxidation of NADPH with *p*-nitroanisole clearly does not occur, even though high rates of mixed-function oxidation of this compound were observed in the phenobarbital-treated rat. The observation that aminopyrine failed to oxidize NADPH in the presence of dinitrophenol, an uncoupling agent of oxidative phosphorylation, is useful in interpreting the apparent paradoxical results obtained with *p*-nitroanisole.

Since [ATP]/[ADP] ratios were significantly lowered by both *p*-nitroanisole alone and the combination of aminopyrine plus dinitrophenol (Table 6), it is likely that both treatments uncouple oxidative phosphorylation. This hypothesis is supported by the structural similarity of dinitrophenol and *p*-nitrophenol. In experiments with isolated rat liver mitochondria (F. C. Kauffman, unpublished work), 0.1 mM-*p*-nitrophenol stimulated succinate-dependent State-3 and State-4 respiration 110 and 85% respectively. Lowering of the [ATP]/[ADP] ratio may be a critical event, because both dehydrogenases of the pentose phosphate pathway are markedly inhibited by ATP (Avigad, 1966; Passonneau *et al.*, 1966; Afolayan, 1972; Kauffman & Johnson, 1970). Inhibitor constants of ATP for glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in liver of phenobarbital-treated rats are 1.8mM and 0.2mM respectively (R. K. Evans & F. C. Kauffman, unpublished work). The inhibitor constant of ATP for glucose 6-phosphate dehydrogenase is increased by P_i (Passonneau *et al.*, 1966). Thus a decrease in the concentration of ATP in the liver by an agent that uncouples oxidative phosphorylation could activate metabolism of hexose phosphate via the oxidative enzymes of the pentose phosphate pathway, which in turn leads to enhanced NADPH generation.

During mixed-function oxidation of a drug such as hexobarbital the rate of NADPH oxidation may exceed the capacity of the liver to regenerate NADPH; therefore the [$NADP^+$]/[NADPH] ratio increases. When an uncoupling agent is either present (e.g. dinitrophenol) or generated (e.g. *p*-nitrophenol), the high capacity of the liver to form NADPH in livers of phenobarbital-treated rats via the pentose phosphate pathway is greater, owing to a loss of adenine nucleotide regulation of both dehydrogenases. The rate of NADPH generation may then either be equal to, or even exceed, the rate of NADPH utilization via mixed-function oxidation. Thus mixed-function oxidation of *p*-nitroanisole to the uncoupling agent, *p*-nitrophenol, ultimately leads to a reduction rather than the expected oxidation of NADP.

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