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MAINTENANCE OF NICOTINAMIDE DINUCLEOTIDE PHOSPHATE CONTENT AND OXIDATION–REDUCTION STATE DURING MIXED-FUNCTION OXIDATION OF *p*-NITROANISOLE IN ISOLATED PERFUSED LIVERS OF VARIOUS SPECIES*

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Abstract—The influence of *p*-nitroanisole, a substrate for mixed-function oxidation, on total $NADP^+$ and NADPH and $NADP^+$/NADPH ratios was examined in perfused livers from three different species. Studies were performed using livers from Sprague–Dawley rats, Syrian golden hamsters and C57BL/6J mice. Although rates of *p*-nitroanisole O-demethylation varied more than 16-fold in perfused livers from these species, $NADP^+$/NADPH ratios calculated from measured concentrations of $NADP^+$ and NADPH and from ratios calculated from substrate pairs assumed to be in near equilibrium with $NADP^+$-dependent dehydrogenases remained remarkably constant under most conditions. Thus, rates of NADPH utilization and generation must be tightly coupled in perfused livers during high rates of mixed-function oxidation. Exceptions to the general pattern noted above occurred in livers of fasted, phenobarbital-treated rats where carbohydrate reserves were depleted and in livers from 3-methylcholanthrene-treated mice where rates of *p*-nitroanisole O-demethylation were exceptionally high. Livers from fed phenobarbital-treated rats displayed a paradoxical decrease in $NADP^+$/NADPH ratios reflecting reduction calculated from substrates assumed to be in near equilibrium with 6-phosphogluconate dehydrogenase during mixed-function oxidation, suggesting that NADPH generation exceeded NADPH utilization in the rat in the fed state. In contrast, the $NADP^+$/NADPH ratio calculated from measured pyridine nucleotides increased in livers of 3-methylcholanthrene-treated mice perfused with *p*-nitroanisole, reflecting oxidation. Moreover, the $NADP^+$/NADPH ratio calculated from substrates assumed to be near equilibrium with 6-phosphogluconate dehydrogenase increased in livers of fasted rats, suggesting that utilization of NADPH exceeded generation. Thus, adequate carbohydrate reserves appear essential for maintenance of NADPH during high rates of mixed-function oxidation.

A wide variety of metabolic processes in intact cells are regulated by the content and oxidation–reduction state of pyridine nucleotides [1–4]. Evidence acquired in our laboratory suggests that a close relationship between NADPH supply and rates of mixed-function oxidation exists in the intact liver [5–7]. Addition of substrates that supply reducing equivalents, e.g. glucose or pyruvate, to perfused livers from fasted rats stimulates *p*-nitroanisole O-demethylation [8]. Rates of *p*-nitroanisole O-demethylation also parallel carbohydrate reserves and do not necessarily correspond to mixed-function oxidase activities measured in isolated microsomes [9]. Another link between mixed-function oxidation and pathways of NADPH generation is suggested by the finding that induction of microsomal mixed-function oxidase systems by phenobarbital is also accompanied by induction of glucose-6-phosphate dehydrogenase [6, 10, 11]. Although these observations suggest that mixed-function oxidation and mechanisms regulating the supply of NADPH are interrelated in intact hepatocytes, the effects of substrates for mixed-function oxidation on the cellular content of NADPH and the oxidation–reduction state of the NADP(H) system have not been evaluated systematically. Because various metabolic events including the generation of reduced glutathione [12] and the biosynthesis of key intermediates such as fatty acids [13] and amino acids [14] are dependent on NADPH, it is important to determine whether alterations in the content of $NADP^+$ and NADPH occur in livers during mixed-function oxidation.

The purpose of the present work was to examine the influence of *p*-nitroanisole, a substrate for the mixed-function oxidase system, on $NADP^+$ and

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NADPH in perfused livers of rats, hamsters and mice, which differ widely in their capacities to oxidize drugs. Accordingly, total $NADP^+$ and NADPH and $NADP^+$/NADPH ratios calculated from substrates assumed to be in near equilibrium with various dehydrogenases were determined in livers of the various species perfused with and without *p*-nitroanisole. Measurements of total $NADP^+$ and NADPH were made to evaluate potential changes in total pools of pyridine nucleotides after exposure of livers to a substrate for mixed-function oxidation. Ratios of $NADP^+$/NADPH were also calculated from selected substrates and products of $NADP^+$-dependent dehydrogenases to estimate the oxidation–reduction state of free intracellular pyridine nucleotides [2, 4, 15–17].

METHODS

Perfusion of rat, mouse and hamster liver. Livers were obtained from female Sprague–Dawley rats (120–140 g), male Syrian golden hamsters (90–100 g), or C57BL/6J mice (25–30 g). Animals treated with phenobarbital received the drug in their drinking water (1 mg/ml) for 2 weeks prior to perfusion experiments to induce the mixed-function oxidase system. Mice treated with 3-methylcholanthrene received one 80 mg/kg injection i.p. 72 hr before the experiment. Livers from all species were perfused with Krebs–Henseleit bicarbonate buffer in a non-recirculating system as described previously [8, 18]. The fluid (37°) was pumped through the liver via a cannula inserted in the vena cava past a Teflon-shielded, Clark-type oxygen electrode. *p*-Nitroanisole *O*-demethylase activity was calculated from concentrations of *p*-nitrophenol and *p*-nitrophenol conjugates in the effluent perfusate as described previously [8, 19].

Determination of pyridine nucleotides and metabolites. Pyridine nucleotides and metabolites were measured in samples of liver that were freeze-clamped 26 min after initiation of perfusion. $NADP^+$ and NADPH were determined immediately after preparation of dilute alkaline extracts of frozen samples employing enzymatic cycling techniques [20, 21]. Cysteine (0.5 mM) was added to prevent oxidation of reduced pyridine nucleotides during the extraction procedure. Total $NADP^+$ and NADPH were measured in aliquots of extracts that were not heated. NADPH was measured in aliquots of the dilute alkaline extracts that were heated for 10 min at 60° to destroy oxidized $NADP^+$ [20, 22, 23]. $NADP^+$ was calculated by subtracting measured NADPH from total $NADP^+$ plus NADPH. Under conditions used in these assays, namely extraction of the pyridine nucleotides in 0.02 N NaOH containing 5 mM cysteine at 0°, there was essentially no loss of $NADP^+$ in the unheated samples. Recovery of $NADP^+$ and NADPH added to tissue extracts and carried through the entire analytical procedure was 94 and 98% respectively. Metabolites were assayed in 0.3 M $HClO_4$ extracts of frozen liver as described previously [6, 24].

$NADPH^+$/NADPH ratios were calculated from tissue concentrations of malate, pyruvate, 6-phosphogluconate, ribulose-5-phosphate, isocitrate and α-ketoglutarate and equilibrium constants of 34.4, 170 and 1010 mM for malic enzyme [4, 17], 6-phosphogluconate dehydrogenase [25], and $NADP^+$-dependent isocitrate dehydrogenase [4] respectively. The calculations assume an intracellular pH of 7.0 and a CO_2 concentration of 1.16 mM [4].

Statistical evaluations were made using Student's *t*-test.

RESULTS

p-Nitroanisole O-demethylation in perfused livers from rats, mice and hamsters. Maximal rates of *p*-nitroanisole O-demethylation determined in perfused livers from Sprague–Dawley rats, Syrian hamsters, and C57BL/6J mice are shown in Table 1. Rates were 5-fold higher in livers of hamsters and about 6-fold higher in livers of C57BL/6J mice than in livers of untreated rats. Although basal rates differed markedly in livers of rats, hamsters and mice, the responses of all three species to phenobarbital treatment were quite similar; rates of *p*-nitroanisole O-demethylation were increased 2.7- to 2.8-fold after exposure of all groups to phenobarbital. Treatment of mice with 3-methylcholanthrene had the same effect as phenobarbital on maximal rates of hepatic mixed-function oxidation.

Oxidized and reduced nicotinamide adenine dinucleotide phosphate content in livers of various species. Data in Table 2 compare $NADP^+$ and NADPH measured in freeze-clamped livers from rats, mice, and hamsters. Total NADP(H) did not vary greatly between the various groups in the absence of *p*-nitroanisole with the exception of the normal C57BL/6J mouse which contained significantly lower ($P < 0.01$) $NADP^+$ and NADPH than the other species. Treatment of mice with 3-methylcholanthrene elevated the total content of $NADP^+$ ($P < 0.01$) and increased $NADP^+$/NADPH ratios ($P < 0.05$).

Addition of *p*-nitroanisole to the perfusion medium did not alter $NADP^+$/NADPH ratios based on measured pyridine nucleotides in any of the species studied except in 3-methylcholanthrene-treated mice (Table 2). The increase in the ratio in livers of 3-methylcholanthrene-treated mice occurred in the absence of a change in the total content of $NADP^+$ plus NADPH. *p*-Nitroanisole also lowered NADPH in livers from hamsters; however, this decline was accompanied by a significant decrease in the total content of $NADP^+$ plus NADPH.

Calculated $NADP^+$/NADPH ratios. Data in Table 3 compare $NADP^+$/NADPH ratios calculated from substrate pairs assumed to be in near equilibrium with several $NADP^+$-dependent dehydrogenases in livers from the three species studied. These ratios, which are frequently assumed to reflect ratios of free oxidized and reduced pyridine nucleotides [1, 3], were considerably lower than ratios based on measured $NADP^+$ and NADPH (Table 2), suggesting that a relatively greater proportion of $NADP^+$ than NADPH was bound.

Pretreatment with phenobarbital produced a significant increase in the $NADP^+$/NADPH ratio

Table 1. Rates of *p*-nitroanisole O-demethylation in perfused livers from various species

Species	*p*-Nitrophenol formation (μmoles·g^{-1}·hr^{-1})
Rat	
Normal (10)	2.7 ± 0.3
Phenobarbital-treated (47)	7.5 ± 0.8
Syrian Hamster*	
Normal (10)	13.2 ± 0.7
Phenobarbital-treated (11)	37.2 ± 5.5
C57BL/6J Mouse	
Normal (12)	16.5 ± 2.9
3-Methylcholanthrene-treated (12)	41.1 ± 4.7
Phenobarbital-treated (5)	44.4 ± 3.4

Values are averages ± SEM for maximal rates of free and conjugated *p*-nitrophenol formed during perfusion of livers from fed animals with 0.2 mM *p*-nitroanisole.

* From Reinke *et al.* [26].

calculated from substrate pairs for 6-phosphogluconate dehydrogenase in the rat ($P < 0.001$) and 3-methylcholanthrene treatment increased this ratio in mouse liver ($P < 0.05$). A significant increase in this ratio calculated from substrates assumed to be in near equilibrium with isocitrate dehydrogenase also occurred in livers of rats treated with phenobarbital ($P < 0.001$). Ratios calculated from substrates for the three dehydrogenases were consistently one to two orders of magnitude lower than those based on measured $NADP^+$ and NADPH. Moreover, there was no correlation ($r < 0.4$) between ratios determined from measured pyridine nucleotides and ratios calculated from substrate pairs. Values calculated from substrates for isocitrate dehydrogenase and "malic" enzyme in perfused livers from normal fed rats are comparable to values calculated from substrates in rat liver *in vivo* [27].

Perfusion of livers with *p*-nitroanisole did not increase $NADP^+$/NADPH ratios calculated from 6-phosphogluconate dehydrogenase in livers from any of the species studied; however, the ratio decreased in livers of phenobarbital-treated rats, reflecting a more reduced metabolic state (Table 3). The ratio calculated from substrates assumed to be in near equilibrium with "malic" enzyme did not change in a consistent manner during perfusion with *p*-nitroanisole; it decreased in livers of normal rats and increased in livers of phenobarbital-treated rats. Although rates of mixed-function oxidation were highest in livers of hamsters and mice, ratios calculated from either malic enzyme or 6-phosphogluconate dehydrogenase did not change significantly during perfusion with *p*-nitroanisole.

Influence of p-*nitroanisole on adenine nucleotides in perfused livers.* Earlier work from our laboratory using perfused livers from phenobarbital-treated rats showed that cellular ATP content is decreased by *p*-nitroanisole possibly via uncoupling of oxidative phosphorylation [6, 24]. Pretreatment of rats with phenobarbital increased the content of ATP and ATP/ADP ratios significantly (Table 4). Perfusion of livers from either normal or phenobarbital-treated rats with *p*-nitroanisole increased ADP and AMP significantly; however, only livers from phenobarbital-treated rats showed significant declines in ATP and ATP/ADP ratios. In contrast to the rat, treatment with inducing agents or perfusion with *p*-nitroanisole did not alter ATP/ADP ratios in livers from mice or hamsters.

Effect of nutritional state on $NADP^+$/NADPH ratios in rat liver. Generation of cytosolic reducing equivalents in the form of NADPH has been suggested to occur primarily via the oxidative enzymes of the pentose phosphate pathway in rat liver [28]. Depletion of substrate for the pentose phosphate pathway lowers rates of NADPH generation about 5-fold [29]. To test the effect of flux changes in the pentose phosphate pathway on pyridine nucleotides, we examined the influence of fasting on $NADP^+$ and NADPH in livers of rats perfused in the presence and absence of *p*-nitroanisole. Fasting increased ratios calculated from measured $NADP^+$ and NADPH in livers from phenobarbital-treated rats but not in livers from normal rats (Table 5). In contrast, ratios calculated from substrates assumed to be in near equilibrium with 6-phosphogluconate dehydrogenase decreased 10-fold in livers from fasted, phenobarbital-treated rats (Table 5). Comparable changes in ratios calculated from malic enzyme and isocitrate dehydrogenase have been noted previously in normal rats starved for 48 hr [4].

Perfusion of liver from fasted normal rats with *p*-nitroanisole increased the $NADP^+$/NADPH ratio calculated from measured pyridine nucleotides (Table 5). In livers from fasted, phenobarbital-treated rats which contain little glycogen [30], infusion of *p*-nitroanisole increased the $NADP^+$/NADPH ratio calculated from 6-phosphogluconate dehydrogenase nearly 5-fold (Table 5). This increase contrasts dramatically with the decrease observed in livers from fed, phenobarbital-treated rats (Table 5).

DISCUSSION

Rates of *p*-nitroanisole O-demethylation ranged between 3 μmoles·g^{-1}·hr^{-1} in livers of normal, fed rats to 44 μmoles·g^{-1}·hr^{-1} in livers from phenobarbital-treated mice in this study (Table 1). Because mixed-function oxidation requires one mole

Table 2. Influence of *p*-nitroanisole on oxidized and reduced nicotinamide adenine dinucleotide phosphate content in perfused livers

Species	$NADP^+$	NADPH	$NADP^+$ plus NADPH	$NADP^+$/NADPH ×100
		(μmoles/kg wet tissue)		
Normal rat				
Control (4)	107.3 ± 16.9	273.6 ± 32.0	381 ± 38	40.3 ± 6.7
0.2 mM *p*-Nitroanisole (4)	76.5 ± 26.6	249.6 ± 32.4	326 ± 21	36.1 ± 17.3
Phenobarbital-treated rat				
Control (8)	104.2 ± 16.3	229.5 ± 18.8	334 ± 22	48.5 ± 7.9
0.2 mM *p*-Nitroanisole (11)	96.7 ± 18.7	268.1 ± 12.1	365 ± 21	37.3 ± 7.4
Hamster				
Control (4)	154.4 ± 16.4	186.2 ± 12.9	341 ± 28	82.6 ± 6.0
0.2 mM *p*-Nitroanisole (6)	117.9 ± 16.0	112.6 ± 13.4*	230 ± 22†	113.2 ± 18.9
Normal C57BL/6J mouse				
Control (4)	34.5 ± 11.2	181.1 ± 16.8	214 ± 15	19.2 ± 7.2
0.2 mM *p*-Nitroanisole (10)	29.8 ± 5.5	206.3 ± 11.6	236 ± 11	14.8 ± 2.7
Phenobarbital-treated C57BL/6J mouse				
Control (4)	99.1 ± 24.4	282.4 ± 81.8	382 ± 92	36.8 ± 3.8
0.2 mM *p*-Nitroanisole (5)	147.2 ± 17.9	244.5 ± 27.3	392 ± 21	64.5 ± 14.1
3-Methylcholanthrene-treated C57BL/6J mouse				
Control (5)	150.2 ± 20.2	247.8 ± 25.1	398 ± 29‡	64.0 ± 12.3§
0.2 mM *p*-Nitroanisole (4)	269.1 ± 36.5†	172.1 ± 18.2†	441 ± 39	163.3 ± 28.7*

Values are averages ± SEM of the number of livers indicated in parentheses. All measurements were made on livers from fed animals. Livers were perfused with *p*-nitroanisole for 6 min prior to freeze-clamping. Oxidized and reduced pyridine nucleotides were determined as described in Methods.

* $P < 0.01$ (control vs *p*-nitroanisole).

† $P < 0.05$ (control vs *p*-nitroanisole).

‡ $P < 0.01$ (treated vs normal).

§ $P < 0.05$ (treated vs normal).

Table 3. Calculated [NADP+]/[NADPH] ratios in perfused livers from rats, hamsters, and mice

Species	[NADP+]/[NADPH] ratio (×100) calculated from: "Malic" enzyme	6-Phosphogluconate dehydrogenase	Isocitrate dehydrogenase
Normal rat			
Control	3.87 ± 0.73 (20)	0.66 ± 0.23 (10)	1.84 ± 0.33 (19)
0.2 mM *p*-Nitroanisole	2.35 ± 0.33* (30)	0.77 ± 0.14 (22)	1.37 ± 0.24 (31)
Phenobarbital-treated rat			
Control	3.79 ± 0.44 (41)	2.95 ± 0.44† (16)	4.04 ± 0.59 (17)
0.2 mM *p*-Nitroanisole	6.36 ± 0.81‡ (32)	1.73 ± 0.34* (14)	2.80 ± 0.36 (16)
Hamster			
Control	0.57 ± 0.05 (8)	0.59 ± 0.17 (8)	
0.2 mM *p*-Nitroanisole	0.48 ± 0.07 (10)	0.45 ± 0.09 (10)	
Normal C57BL/6J mouse			
Control	2.79 ± 0.45 (4)	0.82 ± 0.13 (5)	
0.2 mM *p*-Nitroanisole	5.06 ± 0.84 (10)	0.92 ± 0.19 (4)	
3-Methylcholanthrene-treated C57BL/6J mouse			
Control	3.12 ± 0.47 (5)	3.14 ± 1.19§ (4)	
0.2 mM *p*-Nitroanisole	3.34 ± 0.81 (4)	1.02 ± 0.16 (8)	

Values are averages ± SEM of the number of livers shown in parentheses and are based on metabolites measured in livers that were freeze-clamped 6 min after perfusion with 0.2 mM *p*-nitroanisole.
* $P < 0.05$ (control vs *p*-nitroanisole).
† $P < 0.001$ (treated vs normal).
‡ $P < 0.01$ (control vs *p*-nitroanisole).
§ $P < 0.05$ (treated vs normal).

Table 4. Effect of *p*-nitroanisole on adenine nucleotides in perfused livers from various species

Species	ATP	ADP	AMP	ATP + ADP + AMP	ATP/ADP
	(μmoles/kg wet weight)				
Normal rat					
Control (11)	1605 ± 174	590 ± 50	292 ± 46	2487 ± 188	2.89 ± 0.37
0.2 mM *p*-Nitroanisole (10)	1606 ± 241	756 ± 61*	445 ± 41*	2807 ± 267	2.09 ± 0.26
Phenobarbital-treated rat					
Control (45)	2183 ± 78†	590 ± 28	278 ± 27	3051 ± 82†	3.95 ± 0.22‡
0.2 mM *p*-Nitroanisole (29)	1839 ± 106§	751 ± 38‖	435 ± 47§	3025 ± 94	2.61 ± 0.20‖
Hamster					
Control (4)	1998 ± 169	1124 ± 17	184 ± 32	3306 ± 169	1.78 ± 0.17
0.2 mM *p*-Nitroanisole (5)	2188 ± 297	1302 ± 60*	349 ± 68	3839 ± 337	1.66 ± 0.17
Corn oil-treated mouse					
Control (5)	2706 ± 214	845 ± 169	237 ± 62	3788 ± 411	3.47 ± 0.40
0.2 mM *p*-Nitroanisole (10)	2814 ± 147	829 ± 30	198 ± 15	3840 ± 165	3.42 ± 0.18
3-Methylcholanthrene-treated mouse					
Control (5)	2530 ± 414	641 ± 44	192 ± 31	3363 ± 409	3.96 ± 0.68
0.2 mM *p*-Nitroanisole (4)	2715 ± 138	736 ± 60	182 ± 15	3633 ± 176	3.72 ± 0.17
Phenobarbital-treated mouse					
Control (4)	2818 ± 134	863 ± 76	156 ± 29	3837 ± 99	3.36 ± 0.41
0.2 mM *p*-Nitroanisole (5)	2978 ± 126	863 ± 36	147 ± 17	3989 ± 156	3.46 ± 0.12

Values are expressed as means ± SEM for the number of livers in parentheses.
* $P < 0.05$ (control vs *p*-nitroanisole).
† $P < 0.01$ (phenobarbital-treated rat control vs normal rat control).
‡ $P < 0.05$ (phenobarbital-treated rat control vs normal rat control).
§ $P < 0.01$ (control vs *p*-nitroanisole).
‖ $P < 0.001$ (control vs *p*-nitroanisole).

Table 5. Effect of *p*-nitroanisole on $NADP^+/NADPH$ ratios in perfused livers from fed and fasted normal and phenobarbital-treated rats

	$NADP^+/NADPH \times 100$ ratio calculated from: Measured $NADP^+$ and NADPH		6-Phosphogluconate dehydrogenase	
	Control	0.2 mM *p*-Nitroanisole	Control	0.2 mM *p*-Nitroanisole
Normal rat				
Fed	40.3 ± 6.7 (4)	36.1 ± 17.3 (4)	0.66 ± 0.23 (10)	0.77 ± 0.14 (22)
Fasted	59.5 ± 2.2 (5)	143.1 ± 28.0* (3)		
Phenobarbital-treated rat				
Fed	48.5 ± 7.9 (8)	37.3 ± 7.4 (11)	2.95 ± 0.44 (16)	1.73 ± 0.34† (14)
Fasted	245.6 ± 59.2‡ (8)	324.7 ± 44.4 (19)	0.26 ± 0.04 (9)	1.32 ± 0.46* (10)

Values are averages ± SEM of the number of livers shown in parentheses. Perfusion of livers and analyses of metabolites were as described in Tables 2 and 3.

* $P < 0.01$ (control vs *p*-nitroanisole).

† $P < 0.05$ (control vs *p*-nitroanisole).

‡ $P < 0.01$ (fasted vs fed).

of NADPH to oxidize one mole of substrate, rates of NADPH utilization have varied over at least 16-fold in the various livers studied. Despite this large variation in rates of NADPH utilization for mixed-function oxidation, total NADPH and ratios of $NADP^+/NADPH$ remained remarkably constant. It was only when rates of mixed-function oxidation exceeded 40 $\mu moles \cdot g^{-1} \cdot hr^{-1}$, e.g. in livers of 3-methylcholanthrene-treated mice, that a significant increase in the $NADP^+/NADPH$ ratio, reflecting oxidation, was observed (Table 1). Based on measured NADPH and rates of *p*-nitroanisole O-demethylation in perfused livers, NADPH would be consumed totally in 6.5 min in livers from normal rats and in 0.3 min in livers from 3-methylcholanthrene-treated mice during mixed-function oxidation. Since the largest fraction of NADPH is bound in intact cells [1, 3, 31], the time required to totally deplete free NADPH during mixed-function oxidation would be considerably less than 0.3 to 6.5 min if mechanisms to sustain NADPH generation were not maintained. Thus, rates of NADPH turnover must vary substantially among livers of the various species, and NADPH utilization must be balanced by NADPH generation under most conditions.

Ah-locus responsive mice (C57BL/6J) treated with 3-methylcholanthrene were unable to maintain steady-state concentrations of NADPH during high rates of *p*-nitroanisole O-demethylation. Following *p*-nitroanisole infusion there was a significant oxidation of the total pool of NADPH as indicated by increases in $NADP^+/NADPH$ calculated from measured amounts of $NADP^+$ and NADPH (Table 2). The different effects of *p*-nitroanisole on the oxidation–reduction state of $NADP^+/NADPH$ in livers of phenobarbital and 3-methylcholanthrene-treated mice are noteworthy. Although maximal rates of mixed-function oxidation were the same in both groups (Table 1), a significant oxidation of steady-state amounts of NADPH occurred only in livers of the latter group. The reason for this disparity is unknown but may be related to the capacity of 3-methylcholanthrene-treated mice to maintain high rates of mixed-function oxidation for long periods of time [5]. In contrast to the maintenance of high rates of mixed-function oxidation for extended periods of time in livers of Ah-locus mice treated with 3-methylcholanthrene, maximal rates of mixed-function oxidation were maintained for only a few mintues in liver of this species treated with phenobarbital [5]. Thus, treatment of Ah-locus responsive mice with polycyclic aromatic hydrocarbons may, in some way, couple enhanced rates of NADPH turnover to sustained high rates of mixed-function oxidation.

Oxidation–reduction state of free and bound NADP. The disparity between data presented in Table 2, which report the total content of $NADP^+$ and NADPH in livers of the various species studied, and the ratios of "free" $NADP^+$ and NADPH reported in Table 3, which are based on substrates assumed to be in near equilibrium with various $NADP^+$-dependent dehydrogenases, may be explained in several ways. Measurements of total $NADP^+$ and NADPH in liver do not distinguish between pools of pyridine nucleotides which exist in different subcellular compartments. The content of NADPH is about 5-fold higher in the mitochondrial than in the extramitochondrial compartment of liver and the $NADP^+/NADPH$ ratio differs substantially between these compartments [32–34]. The large differences in ratios reported in Table 2 and 3 are in accord with the idea that the distribution of $NADP^+$ and NADPH in different subcellular compartments is not identical.

The ratios reported in Table 3 are also based on the assumption that substrates for the various $NADP^+$-dependent dehydrogenases are in equilibrium with "free" $NADP^+$ and NADPH in specific subcelluar compartments. For example, 6-phosphogluconate dehydrogenase is localized exclusively in the cytosolic compartment [4, 25]. The ratios calculated from substrates for the three dehydrogenase reported in Table 3 differed considerably from each other. Based on these data, it may be concluded that the enzymes and substrates are not distributed evenly in the same subcellular com-

partment, that the substrate pairs are not in equilibrium with "free" $NADP^+$ and NADPH, or both. $NADP^+$-dependent isocitrate dehydrogenase is found in both mitochondria and cytosol [4]. It is likely that substrates for malic enzyme are not in equilibrium with $NADP^+$ and NADPH because of the very low activity of this enzyme. Thus, measurements of the substrates and product of 6-phosphogluconate dehydrogenase probably provide a more reliable index of cytosolic $NADP^+$/NADPH. However, caution must also be used with this indicator because calculated ratios reported in Table 3 are based on measurements of steady-state concentrations of substrates, and it is not known whether these represent true equilibium values. If the substrates or products for any of the enzymes are rapidly metabolized or extruded from the cell, then the discrepancies noted in Table 3 would result from the faulty assumption that the substrates and products are in equilibrium with the indicator enzyme. Based on these considerations, measurements of total content of $NADP^+$ and NADPH probably provide a more reliable index of the oxidation–reduction state of $NADP^+$ and NADPH in the isolated perfused liver.

The finding that $NADP^+$/NADPH ratios calculated from total measured $NADP^+$ and NADPH were consistently much higher (1–2 order of magnitude) than ratios based on substrates assumed to be in near equilibrium with the three $NADP^+$-dependent dehydrogenases suggests that the oxidized form of the pyridine nucleotide in intact cells is preferentially bound. This conclusion is supported by the observation that binding constants for $NADP^+$ for various dehydrogenases range from 1 to 100 μM and are 150–200 times lower than binding constants for NADPH [35–37]. Relationships between bound and free forms of NADPH must differ considerably in livers from the various species since there was no correlation between $NADP^+$/NADPH ratios obtained by direct measurement and those calculated from various substrate pairs (Tables 2 and 3). The greatest disparity between these ratios occurred in hamster liver. This species had the highest $NADP^+$/NADPH ratio from measured values and the lowest ratios calculated from substrate pairs. Thus, hamster liver may have a relatively high binding capacity for $NADP^+$.

Role of carbohydrate reserves and ATP. The importance of an available supply of carbohydrate and the involvement of intracellular effectors in regulating rates of NADPH generation are illustrated by data obtained using perfused livers from fed and fasted rats. Based on the comparatively high activities of the oxidative enzymes of the pentose phosphate pathway in rat liver [6], this route of metabolism serves as a major source of NADPH for mixed-function oxidation. In fact, NADPH is derived from the oxidative enzymes of the pentose phosphate pathway in perfused livers from fed rats at rates which exceed rates of mixed-function oxidation of *p*-nitroanisole by at least 2-fold [29].

Stimulation of metabolism via the pentose phosphate pathway by *p*-nitroanisole likely involves alterations in the turnover of NADPH and decreases in the cellular content of ATP. Both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are inhibited by NADPH [37] and ATP [11]. Events which lower cellular NADPH and ATP will result in disinhibition of the two oxidative enzymes of the pentose pathway and will lead to activation of NADPH synthesis. Interestingly, declines in ATP and ATP/ADP ratios during perfusion with *p*-nitroanisole occurred only in livers of phenobarbital-treated rats (Table 4). These decreases in ATP and ATP/ADP ratios were accompanied by a significant decrease in $NADP^+$/NADPH ratios calculated from substrates assumed to be in near equilibrium with 6-phosphogluconate dehydrogenase (Table 3). There was also a tendency for total $NADP^+$ to decrease and total NADPH to increase (Table 2). Thus, NADPH generation may have exceeded NADPH utilization during perfusion of livers from fed rats with *p*-nitroanisole due to disinhibition of the two oxidative enzymes of the pentose pathway by decreased ATP. Decreases in the ratio calculated from substrates assumed to be in near equilibrium with 6-phosphogluconate dehydrogenase were not noted in livers of the other species in which the ATP content and ATP/ADP ratios did not change.

Results obtained using livers of fasted rats illustrate the importance of carbohydrate supply in maintaining intracellular stores of NADPH. Perfusion of livers from fasted rats with *p*-nitroanisole increased the $NADP^+$/NADPH ratio 2.4-fold (Table 5). This oxidation of NADPH in livers of fasted rats can be explained by a lack of substrate for the oxidative enzymes of the pentose pathway. Since glucose-6-phosphate is reduced markedly in the fasted state, rates of oxidation via glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are diminished greatly [29]. Results obtained in livers of fasted rats contrasted dramatically with those obtained in livers from fed, phenobarbital-treated rats. In livers of fed animals, the $NADP^+$/NADPH ratio actually decreased, indicative of reduction of $NADP^+$ rather than oxidation of NADPH. These data argue strongly that only in the presence of adequate substrates for NADPH-generating enzymes are intracellular stores of hepatic NADPH maintained during high rates of mixed-function oxidation.

The maintenance of NADPH in hepatocytes during acute challenges with a substrate for mixed-function oxidation suggests that maintenance of steady-state levels of NADPH is critical to the metabolic functions and viability of these cells. In addition to serving an important function for biosynthetic events such as fatty acid, lipid, and amino acid biosynthesis, NADPH is also required to maintain intracellular stores of reduced glutathione. Loss of intracellular reduced glutathione produced by a variety of toxic agents [12, 38] is associated with a rapid loss of viability and function of hepatocytes.

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