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STIMULATION OF MIXED-FUNCTION OXIDATION BY NADPH IN PERFUSED MOUSE LIVERS

STUDIES WITH SAPONIN-PERMEABILIZED TISSUE*

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Abstract—In perfused livers from fed and fasted β-naphthoflavone-treated C57BL/6J mice, maximal rates of *p*-nitroanisole O-demethylation were 30–40 μmoles/g/hr and 15–20 μmoles/g/hr respectively. The detergent saponin, at concentrations ranging from 0.001 to 0.005%, was infused between 2 and 30 min to establish optimal conditions to permeabilize plasma membranes. Permeabilization was assessed by release of lactate dehydrogenase and stimulation of *p*-nitroanisole O-demethylation by citrate. Saponin (0.005% for 5 min) alone had little effect on the rates of *p*-nitroanisole O-demethylation or conjugation of *p*-nitrophenol by perfused livers. Further, dicarboxylates or NADPH had no effect on rates of monooxygenation by perfused mouse liver in the absence of saponin. In saponin-treated livers from fasted mice, however, rates of monooxygenation were increased rapidly by infusion of dicarboxylates (10 mM malate, citrate, or isocitrate) or an NADPH-generating system (60 and 110% respectively), over a 6–8 min period. During this time period, cellular energetics were not comprised as reflected by normal rates of glucuronidation of *p*-nitrophenol. Thus, non-permeable metabolites can enter saponin-permeabilized cells in the perfused liver. Rates of monooxygenation were increased 40–60% in livers from fed mice by citrate, NADPH (200 μM) or an NADPH-generating system. In contrast, saponin decreased mixed-function oxidation assayed in isolated microsomes incubated with an NADPH-generating system. Taken together, these data support the hypothesis that maximal rates of monooxygenation in intact hepatocytes from fed as well as fasted mice is limited by the availability of NADPH.

In the presence of excess oxygen and substrate, rates of monooxygenation in intact cells are determined both by the activity of mixed-function oxidase components and the supply of NADPH, which, in turn, can be influenced by the supply of substrates for NADPH-generating enzymes and by reactions competing for NADPH [1]. Carbohydrates which diffuse into the cell readily such as sorbitol, xylitol and glucose stimulate mixed-function oxidation in perfused livers from several species including rats, mice and hamsters [2–5] and in hepatocytes isolated from fasted rats [6] presumably by providing substrates for NADPH-generating enzymes. Based on such experiments, it is generally accepted that NADPH supply is an important rate-determinant of mixed-function oxidation in the fasted state. On the other hand, carbohydrates do not stimulate the oxidative O-demethylation of *p*-nitroanisole in livers from well-fed rats [3] or mice [5]. Thus, either sugars do not increase NADPH in the fed state, or monooxygenation enzymes are saturated with cofactor and do not respond to increases in NADPH supply. Alternatively, increases in NADPH may be used preferentially for other NADPH-consuming processes. From the information available, it is not clear if NADPH supply is a rate-determining factor for mixed-function oxidation in the fed state.

Recently, mild detergent treatment with agents such as saponin, digitonin and Filipin has been used to introduce ionized molecules into cells. Saponin appears to permeabilize cells by forming insoluble complexes with membrane cholesterol [7], leading to an alteration of membrane structure. The purpose of this study was to develop a method to permeabilize cells in perfused livers from mice with saponin, while minimizing possible adverse effects on cellular function. Perfused livers from β-naphthoflavone-treated C57BL/6J mice were employed since they have very high rates of monooxygenation and thus high rates of NADPH utilization (e.g. 40 μmoles/g/hr with *p*-nitroanisole as substrate [5]). After saponin treatment, NADPH and dicarboxylates were added to evaluate the effect of NADPH on monooxygenation in intact cells in the fed state. The data are consistent with the hypothesis that NADPH supply is an important determinant of high rates of monooxygenation in livers from well-fed mice.

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MATERIALS AND METHODS

Treatment of animals. Male C57BL/6J mice (Jackson Laboratories), age 5–15 weeks, were allowed free access to food and tap water or were deprived of food for 20 hr prior to liver perfusion where indicated. All mice were injected (80 mg/kg) intraperitoneally once daily with β-naphthoflavone in corn oil (8 mg/ml) for 3 days.

Mouse liver perfusion. The technique for mouse liver perfusion is explained in detail elsewhere [5]. Krebs–Henseleit bicarbonate buffer, pH 7.4, was saturated with an oxygen/carbon dioxide mixture (95/5) at 37° and was pumped into the portal vein at rates of about 6 ml/min/g of liver. Saponin, dicarboxylates, NADPH and an NADPH-generating system were infused in perfusate at pH 7.4 at concentrations indicated in the figure and table legends. The NADPH-generating system consisted of 6 mg DL-isocitrate, 0.05 units isocitrate dehydrogenase, and 35 μg $NADP^+$ infused into the liver per min.

Analytical measurements. p-Nitroanisole (0.2 mM) dissolved in Krebs–Henseleit bicarbonate buffer was infused as indicated in the figures, and *p*-nitrophenol was monitored spectrophotometrically in the effluent perfusate as described elsewhere [2]. Glucuronide and sulfate conjugates of *p*-nitrophenol were measured enzymatically as described previously [8]. Under these conditions, 80–90% of the conjugates were glucuronides [5]. Rates of monooxygenation were determined from the concentration of *p*-nitrophenol and conjugates in the effluent perfusate, the flow rate, and the liver wet weight. Lactate dehydrogenase in the effluent perfusate was determined by standard enzymatic procedures [9].

Hepatic microsomes were prepared by differential centrifugation [10], and *p*-nitroanisole O-demethylase activity was determined in microsomes as described elsewhere [5].

Histological procedures. Trypan blue (0.2 mM; Sigma Chemical Co., St. Louis, MO) was infused into livers for 10 min following exposure to saponin for various times. Livers were then perfused with 1% paraformaldehyde for 6 min and fixed tissue was embedded in paraffin and processed for light microscopy. Sections were stained with eosin, a cytoplasmic stain, so that trypan blue could be identified in the nuclei of damaged cells [11].

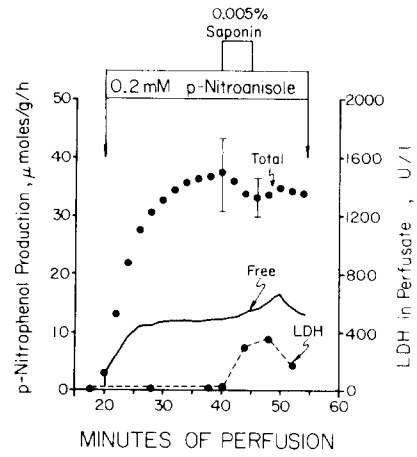

Fig. 1. Effect of saponin on *p*-nitroanisole O-demethylation in perfused liver from fed β-naphthoflavone-treated mice. C57BL/6J mice were treated with β-naphthoflavone in corn oil as described in Materials and Methods. Krebs–Henseleit buffer containing 0.2 mM *p*-nitroanisole was infused as indicated by the arrows and horizontal bars. The optical density of the effluent perfusate was monitored continuously at 436 nm to measure free *p*-nitrophenolate ion (solid line). Samples of effluent perfusate collected every 2 min were incubated with sulfatase and β-glucuronidase to hydrolyze conjugates (total). The difference between total and free *p*-nitrophenolate production represents conjugates of which 80–90% were glucuronides. Lactate dehydrogenase was determined by standard enzymatic procedures (LDH; dotted line). Data represent mean ± S.E.M. from five livers.

RESULTS

Effect of saponin on p*-nitroanisole O-demethylation in perfused mouse livers.* Citrate, a highly-charged anion, does not normally enter cells; there-

Table 1. Effect of saponin on *p*-nitroanisole O-demethylation and lactate dehydrogenase release by perfused mouse liver

Saponin (%)	Nutritional state	Minutes of saponin infusion	Number of livers	*p*-Nitroanisole O-demethylation (μmoles/g/hr) Before saponin	After saponin	Difference	Maximal LDH in perusate (units/liter)
0.001	Fasted	2	3	10.9	12.4	1.5	N.D.*
		20	3	17.0	26.5	9.5	1503
0.005	Fasted	2	5	15.8	32.6	16.8†	885
		5	2	14.3	40.5	26.2	1949
	Fed	2	2	21.4	31.9	10.5	657
		5	5	41.1	64.5	23.4†	1230

Livers from β-naphthoflavone-treated C57BL/6J mice were perfused as described in Materials and Methods. From 20 to 40 min, control rates of *p*-nitroanisole O-demethylation were established prior to infusion of 10 mM citrate. At 50 min, saponin was infused at concentrations and for times indicated in the table. LDH represents maximal release, usually 5 min after saponin infusion was initiated.

* Not detectable.

† $P < 0.01$ for comparisons before and after saponin, using matched-pairs *t*-test.

Table 2. Effects of dicarboxylates and NADPH on *p*-nitroanisole O-demethylation in saponin-treated perfused mouse liver

Saponin (%)	Nutritional state	Addition	N	*p*-Nitroanisole O-demethylation (μmoles/g/hr) Before saponin	After saponin	Difference	% Stimulation
0.005	Fed	None	5	37.5 ± 6.4	33.1 ± 3.5	−4.5 ± 3.0	
	Fed	Citrate	5	41.1 ± 2.8	64.8 ± 5.4	23.7 ± 3.8*	58
	Fed‡	NADPH	9	30.7 ± 4.9	44.2 ± 4.3	13.4 ± 2.3*	44
	Fed	NADPH gen. system	5	34.8 ± 2.6	48.6 ± 4.7	13.8 ± 2.8*	40
	Fasted	None	4	20.4 ± 2.9	21.3 ± 1.6	0.9 ± 1.7	
	Fasted	Citrate	5	15.8 ± 2.0	33.3 ± 3.8	17.5 ± 2.0*	111
	Fasted	NADPH gen. system	5	20.8 ± 1.9	44.2 ± 8.0	23.4 ± 8.8†	112
	Fasted	Isocitrate	5	17.7 ± 3.1	31.2 ± 4.1	13.5 ± 2.1*	76
	Fasted	Malate	5	15.9 ± 1.7	25.3 ± 2.0	9.4 ± 2.1†	60

Livers from β-naphthoflavone-treated C57BL/6J mice were perfused as described in Materials and Methods. At 20 min, *p*-nitroanisole (0.2 mM) infusion was initiated. At 40 min, dicarboxylates (10 mM), NADPH (200 μM) or an NADPH-generating system (see Materials and Methods) was infused. At 50 min, saponin was infused for either 2 (fasted livers) or 5 min (fed livers). Samples of effluent perfusate were collected every 2 min for measurement of free and conjugated *p*-nitrophenol.

* $P < 0.01$ and † $P < 0.05$, compared to the fasted, no addition control.

‡ [Saponin] was 0.01%. Values are means ± S.E.

fore, stimulation of *p*-nitroanisole O-demethylation by citrate was used to establish conditions for permeabilization of the plasma membrane. Stimulation of *p*-nitroanisole O-demethylation occurred when citrate enhanced NADPH formation (Table 1). Saponin (0.001 or 0.005%) was infused for different time periods into livers perfused with *p*-nitroanisole (0.2 mM) in the presence or absence of citrate (10 mM). In the absence of citrate, saponin (0.005%) did not affect the rates of *p*-nitroanisole O-demethylation or conjugation of *p*-nitrophenol in livers from either fed or fasted mice significantly (Fig. 1, Table 2). The tendency for rates to decline slightly (*ca.* 10%) was most likely due to leakage of NADPH from the cell. Further, citrate in the absence of saponin also did not affect rates of monooxygenation (Fig. 2A, 3B). In livers from fasted mice, infusion of 0.001% saponin for up to 10 min caused minimal

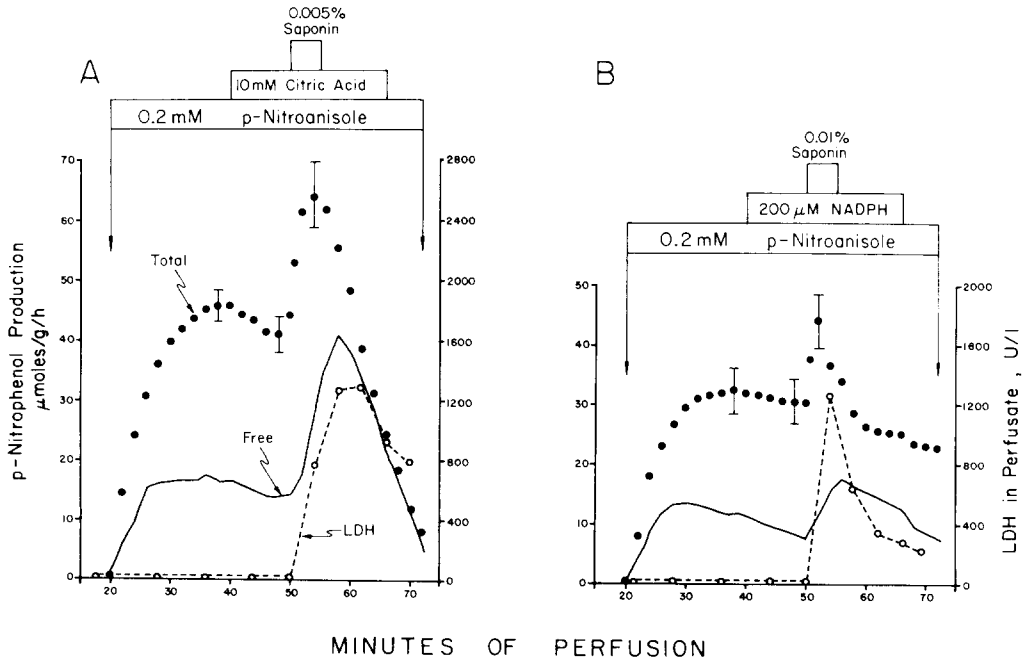

Fig. 2. Effect of citrate and NADPH on *p*-nitroanisole metabolism in perfused liver from fed β-naphthoflavone-treated mice. Experimental conditions were as in Fig. 1, except that saponin was 0.01% in (B). (A) Citrate, 10 mM; five livers. (B) NADPH, 200 μM; nine livers.

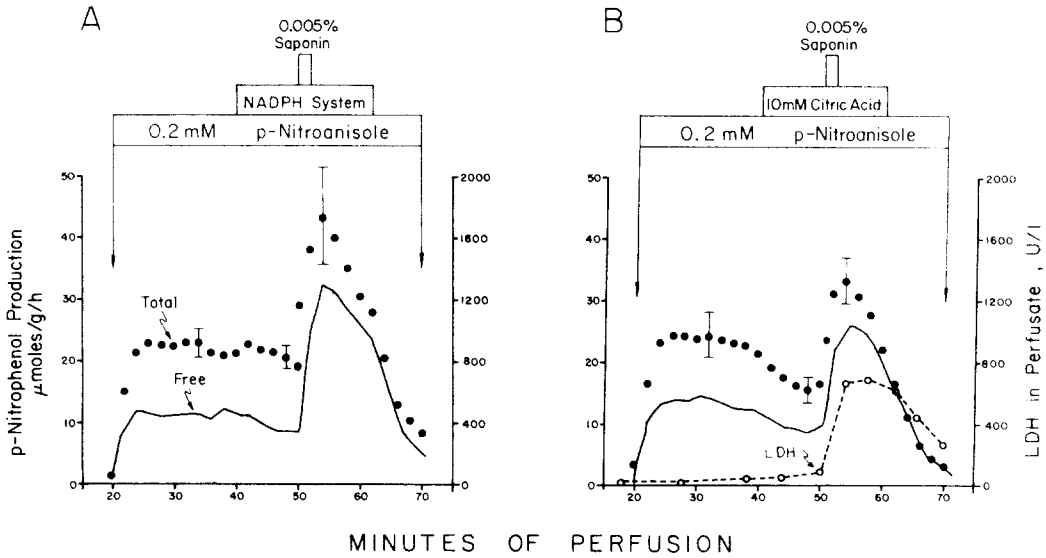

Fig. 3. Effect of NADPH-generating system and citrate on *p*-nitroanisole metabolism in perfused livers from fasted β-naphthoflavone-treated mice. Experimental conditions were as in Fig. 1, except that saponin was infused for 2 min. (A) NADPH-generating system; five livers. (B) Citrate, 10 mM; five livers. LDH could not be determined in the presence of the NADPH-generating system in (A).

release of lactate dehydrogenase and citrate did not affect rates of *p*-nitroanisole O-demethylation (data not shown); however, with infusion of saponin for longer time periods (2–20 min), citrate nearly doubled rates of monooxygenation (Table 1). Under these conditions, lactate dehydrogenase in the effluent perfusate reached values greater than 1000 units/l (Table 1). With higher saponin concentrations (0.005%), stimulation of mixed-function oxidation by citrate and release of lactate dehydrogenase in livers from fasted mice occurred in 2 min. In contrast, 5 min of saponin (0.005%) infusion was required to permeabilize livers from fed mice (Table 1).

Approximately 10% of the cells in periportal areas of the liver lobule were damaged irreversibly when livers from fed mice perfused with saponin (0.005% for 5 min) as reflected by the uptake of trypan blue (data not shown). Exclusive damage to periportal areas has also been achieved by perfusion of livers with the detergent digitonin [12].

Effect of saponin and citrate on microsomal p-nitroanisole O-demethylation. Concentrations of saponin used in perfused livers did not affect *p*-nitroanisole O-demethylation in microsomes incubated with an NADPH-generating system (Table 3). At higher concentrations (0.01%), the detergent diminished rates of *p*-nitroanisole metabolism by about one-third. Citrate alone (10 mM) had no effect on rates of *p*-nitroanisole O-demethylation (Table 3).

Effect of dicarboxylates and NADPH on rates of mixed-function oxidation in perfused livers from fed and fasted mice. In perfused livers from β-naphthoflavone-treated mice, rates of *p*-nitroanisole O-demethylation were between 30 and 40 μmoles/g/hr in the fed state and between 15 and 20 μmoles/g/hr following fasting. These rates are comparable to

Table 3. Effect of saponin on microsomal *p*-nitroanisole O-demethylase activity

	p-Nitroanisole O-demethylation (nmoles/min/mg protein)		
Addition	*p*-Nitrophenol (I)	4-Nitrocatechol (II)	Total (I + II)
None	3.49	0.42	3.91
Citrate, 10 mM	3.21	0.32	3.53
Saponin			
0.001%	2.67	0.24	2.91
0.001%	3.43	0.35	3.78
0.01%	2.06	0.09	2.15

Microsomes were prepared by standard differential centrifugation techniques, and *p*-nitroanisole O-demethylation was determined as described in Materials and Methods. All incubations except those labeled "None" contained 10 mM citrate. Data represent averages from triplicate determinations on microsomes pooled from six β-naphthoflavone-treated mice.

results obtained previously [5]. The infusion of citrate, NADPH or an NADPH-generating system did not alter rates of *p*-nitroanisole O-demethylation in the absence of saponin (Figs. 2 and 3). These results contrast sharply with the stimulation of mixed-function oxidation by 40–60% in livers from fed mice (Fig. 2) when citrate and NADPH were infused in the presence of saponin. During saponin infusion, maximal rates of glucuronidation of *p*-nitrophenol were maintained for 6–8 min; 10–12 min after initiation of saponin infusion, however, both mixed-function oxidation and glucuronidation were decreased dramatically (Figs. 2 and 3). Lactate dehydrogenase was released into the effluent perfusate following the stimulation of monooxygenation (Fig. 2; Table 1).

In saponin-permeabilized livers from fasted mice, citrate and the NADPH-generating system stimulated *p*-nitroanisole O-demethylation by about 110%. Addition of isocitrate or malate to the perfusate under these conditions also stimulated mixed-function oxidation in livers from fasted mice 60–80% (Fig. 2; Table 2).

DISCUSSION

Permeabilization of perfused mouse liver with saponin. Considerable progress has been made in the last decade in permeabilizing cellular membranes. The polyene antibiotic complex Filipin renders cells permeable by forming multimolecular complexes with plasma membrane cholesterol [13] without affecting mitochondrial function [14]. Cholesterol also forms insoluble complexes with the detergent saponin [7] which has been used extensively to permeabilize muscle fibers, chromaffin cells and hepatocytes [15–17]. In addition, mild digitonin treatment has been used to permeabilize isolated hepatocytes [18] and perfused livers [12].

In the present study, we found that 2-min pulses of saponin (0.005%) permeabilized livers from fasted mice, whereas 5 min were required in livers from fed mice (Table 1). Under these conditions, LDH was released and about 10% of the cells were damaged irreversibly as indicated by trypan blue uptake at the end of the experiment. Three lines of evidence suggest that transient, mild treatment with saponin did not alter hepatocyte viability dramatically. First, mixed-function oxidation was not inhibited in perfused mouse livers by infusion of 0.005% saponin (Fig. 1; Table 2), suggesting that monooxygenase components and cellular NADPH-generating systems were unaffected. Second, since glucuronidation is sensitive to alterations in the intracellular energy status and pyridine nucleotide redox state [19, 20], the fact that it was not altered during the initial 10 min of saponin infusion indicates that the detergent did not seriously compromise cellular energy status. Third, over 90% of the hepatocytes excluded trypan blue under these conditions. Thus, mild treatment with saponin (0.005%) does not affect hepatocyte viability dramatically yet permeabilizes the cells as reflected by the stimulation of monooxygenation by citrate or NADPH and ultimately release of lactate dehydrogenase.

Stimulation of mixed-function oxidation in perfused mouse liver by NADPH. Thurman and Scholz [21] showed that respiration of perfused livers from fasted rats is increased markedly after infusion of aminopyrine, a substrate for the mixed-function oxidation system. Increases were not observed, however, when the experiment was performed in the liver of a fasted rat in the presence of an inhibitor of the mitochondrial respiratory chain, antimycin A. On the other hand, hepatic microsomes incubated in the presence and absence of antimycin A oxidize aminopyrine at similar rates [1] when supplied with an active NADPH-generating system. These data suggested that NADPH supply was a major rate-determinant for mixed-function oxidation in livers from fasted rats. Furthermore, in the fasted state, addition of sorbitol, xylitol or glucose, substrates which increase NADPH supply, stimulates mixed-function oxidation in perfused livers [2–5] and isolated hepatocytes [6]. Thus, it is generally accepted that NADPH supply is rate-limiting for monooxygenation in the fasted state.

In the fed state, the role of NADPH supply for monooxygenase reactions is less clear. High rates of *p*-nitrophenol production by perfused livers from phenobarbital-treated rats [2], normal C57BL/6J mice [5] or normal hamsters [4] are linear for less than 2 min and then decline rapidly to 25–50% of control values. Since oxygen and substrate were supplied in excess, a decline in NADPH supply most likely accounted for the transient decrease in rates of mixed-function oxidation under these conditions. These observations suggest that NADPH-generating systems cannot keep pace with rates of NADPH utilization when rates of monooxygenation are high. In support of this hypothesis, we observed that perfused livers from Ah locus-responsive mice treated with 3-methylcholanthrene or β-naphthoflavone maintained high rates of monooxygenation for long periods of time; however, rates decline with time in livers from 3-methylcholanthrene-treated, Ah locus-nonresponsive mice (e.g. they resembled untreated mice). Thus, based on the kinetics of *p*-nitroanisole O-demethylation, it was concluded that 3-methylcholanthrene treatment of Ah locus-responsive mice stimulates not only P_1-450 synthesis but also increases the ability of the liver to supply NADPH at high rates [5].

In spite of the above kinetic information suggesting rate limitation by NADPH in the fed state, carbohydrate infusion does not affect rates of mixed-function oxidation of *p*-nitroanisole in livers from fed rats [3] or mice [5]. Two possible interpretations of these data exist: either NADPH is in excess and not a major rate-determinant for monooxygenation in the fed state, or carbohydrates do not increase NADPH supply for mixed-function oxidation in the fed state.

Insight into this problem is provided with saponin-treated livers in the studies reported here. Mixed-function oxidation of *p*-nitroanisole was stimulated 40–60% by NADPH or citrate in livers from fed, β-naphthoflavone-treated Ah locus-responsive mice permeabilized with saponin (Table 2, Fig. 2). This was not an effect of saponin on mixed-function oxidase components *per se*, since saponin alone tended to diminish rates of monooxygenation in isolated microsomes (Table 3) and in perfused livers (Fig.

1). These data indicate that NADPH can indeed stimulate mixed-function oxidation in livers from fed mice. It is possible, therefore, that NADPH supply from the pentose cycle is maximal in the fed state and is not increased by infusion of carbohydrates. This conclusion is supported by the observation that glucose (10 mM) does not alter rates of pentose cycle flux in livers from fed, phenobarbital-treated rats [22].

The K_m for NADPH-cytochrome P-450 reductase is below measured contents of total NADPH in the cell [23]; therefore, it is curious that increases in NADPH stimulated mixed-function oxidation in saponin-treated livers. However, NADPH is bound to various dehydrogenases and is localized predominantly in the mitochondria [24] in intact cells. Further, many NADPH-utilizing systems (e.g. fatty acid synthesis, flavin-linked monooxygenation, GSSG reduction) utilize NADPH in intact cells. Thus, it is reasonable to assume that the concentration of free NADPH available for monooxygenation in intact cells is extremely low. The observation that addition or generation of NADPH in detergent-treated livers stimulated monooxygenation in perfused mouse livers from β-naphthoflavone-treated mice (Fig. 2) is consistent with the hypothesis that free intracellular NADPH concentrations are rate-limiting for high rates of cytochrome P-450-dependent monooxygenation in the fed state. In support of this hypothesis, NADPH also stimulates benzo[*a*]pyrene metabolism in permeabolized rat hepatoma cells [25].

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