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OXIDATION–REDUCTION STATE OF FREE NADP$^+$ DURING MIXED-FUNCTION OXIDATION IN PERFUSED RAT LIVERS—

EVALUATION OF THE ASSUMPTIONS OF NEAR EQUILIBRIUM BY COMPARISONS OF SURFACE FLUORESCENCE CHANGES AND CALCULATED NADP$^+$:NADPH RATIOS*

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Abstract—Changes in cellular pyridine nucleotide and flavoprotein oxidation-reduction states associated with mixed-function oxidation of *p*-nitroanisole, hexobarbital and aminopyrine by perfused rat livers were studied. Surface fluorescence techniques were compared with NAD(P)$^+$/NAD(P)H ratios calculated from substrates assumed to be in near equilibrium with various dehydrogenases in freeze-clamped liver samples. *p*-Nitroanisole and *p*-nitrophenol caused a large decrease in pyridine nucleotide (366 → 450 nm) fluorescence as a result of fluorescence quenching. This decrease, therefore, did not reflect oxidation of pyridine nucleotides. Moreover, *p*-nitroanisole infusion decreased the free NADP$^+$:NADPH ratios calculated from malic enzyme and isocitrate dehydrogenase. Hexobarbital, which did not produce fluorescence quenching, caused an oxidation in pyridine nucleotides as indicated by both a decrease in surface fluorescence and an increase in the calculated NADP$^+$/NADPH ratio. These data indicate that free NADP$^+$/NADPH ratios calculated from substrates, which are assumed to be in near equilibrium with NADPH-generating enzymes, indeed reflect the NADPH redox state in intact liver cells.

Maximal rates and kinetics of mixed-function oxidation of drugs and other xenobiotics are often different in microsomes and whole cell preparations isolated from the same tissue. These differences are in all likelihood due to regulating factors, such as NADPH supply, which may be rate limiting in whole cells. During the process of mixed-function oxidation, equimolar amounts of NADPH and drug substrate are oxidized [1].

The pyridine nucleotide redox state may be monitored noninvasively during mixed-function oxidation with surface fluorescence techniques in the perfused liver and may also be calculated from substrates assumed to be in near equilibrium with various dehydrogenases *in situ*. For example, during metabolism of hexobarbital by the perfused rat liver, an increase (oxidation) in the NADP$^+$/NADPH ratios, calculated from the substrate and product of malic enzyme measured in freeze-clamped tissues, occurred [2]. This change was accompanied by a decrease in pyridine nucleotide fluorescence (oxidation) from the surface of the perfused liver. On the other hand, the predicted oxidation is not always detected with these methods. During the mixed-function oxidation of *p*-nitroanisole, oxidation of the NADP$^+$/NADPH ratio was not observed in perfused livers from fed, phenobarbital-treated rats [3].

The criteria for calculation of free NADP$^+$/NADPH ratios are based on the assumption that near equilibrium conditions exist between the oxidized and reduced substrates of highly active NADP$^+$-dependent dehydrogenases [4]. The observation that the NADP$^+$/NADPH ratio calculated from malic enzyme was unchanged during *p*-nitroanisole metabolism [3] suggests either that near equilibrium conditions do not exist in the hepatocyte, or that NADPH biosynthesis is stimulated under these conditions to offset NADPH oxidation.

The experiments described in this report compare the effects of several substrates for mixed-function oxidation on both the calculated NADP$^+$/NADPH ratio and the pyridine nucleotides detected directly by surface fluorescence. Both techniques reflect oxidation of pyridine nucleotides during hexobarbital metabolism. On the other hand, *p*-nitroanisole and aminopyrine modify surface fluorescence of pyridine nucleotides by fluorescence quenching or alterations of the NAD$^+$ system respectively. Because surface fluorescence and calculated ratios indicate an oxidation in NADP$^+$ systems in the presence of hexobarbital, it is concluded that both methods indicate the redox state of the intracellular free NADP$^+$.

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MATERIALS AND METHODS

Animals. Female Sprague–Dawley rats received sodium phenobarbital (1 mg/ml) in drinking water for 2 weeks prior to perfusion experiments to induce the microsomal mixed-function oxidase enzymes [5].

Liver perfusion. Details of the perfusion technique have been described elsewhere [6, 7]. Livers were perfused with Krebs–Henseleit bicarbonate buffer [8] (pH 7.4) saturated with an oxygen–carbon dioxide mixture (95:5) in a non-recirculating system. The fluid (37°) was pumped via a cannula in the vena cava past a teflon-shielded oxygen electrode before being discarded. Rates of oxygen uptake were calculated from the liver weight, the flow rate, and the arterial-venous oxygen concentration difference. *p*-Nitroanisole (0.2 mM) was dissolved in Krebs–Henseleit bicarbonate buffer, and the continuous formation of the *p*-nitrophenolate ion was monitored spectrally as described previously [9]. Aminopyrine, hexobarbital, metyrapone and *p*-nitrophenol were dissolved in distilled water and infused into the perfusion fluid entering the liver at the final concentrations indicated in the text and figure legends.

Metabolite measurement. Metabolites were measured in $KClO_4$ extracts of livers that had been freeze-clamped with tongs chilled in liquid nitrogen [10]. Samples of frozen liver weighing about 200 mg were powdered and extracted with 0.3 M $HClO_4$ as described previously [11]. The protein-free extracts were neutralized with 2 M $KHCO_3$ and stored at −80° until assayed for metabolites.

Intermediates of the Embden–Meyerhoff pathway were measured enzymatically by fluorometric procedures described by Lowry and Passonneau [12]. Intermediates of the tricarboxylic acid cycle were measured by the methods of Goldberg *et al.* [13]. Cytoplasmic $(NADP^+)/(NADPH)$ ratios were calculated as described previously [4].

Surface fluorescence of pyridine nucleotides and flavoproteins. The redox state of a tissue may be monitored noninvasively employing surface fluorescence techniques. Certain oxidized flavoproteins fluoresce at 520 nm, when excited at 460 nm, while reduced pyridine nucleotide fluorescence is excited at 366 nm and monitored at 450 nm [14, 15]. Changes in flavoprotein fluorescence reflect changes predominately in the mitochondrial oxidation-reduction state [15], while pyridine nucleotide fluorescence indicates changes in both mitochondrial and extramitochondrial NAD^+ and $NADP^+$ coenzymes [15, 16]. With this technique, the liver is alternately illuminated with pulses of 366 and 460 nm light. The emitted fluorescence is detected by the photomultiplier after passing through secondary filters having transmission maxima at 450 and 520 nm respectively.

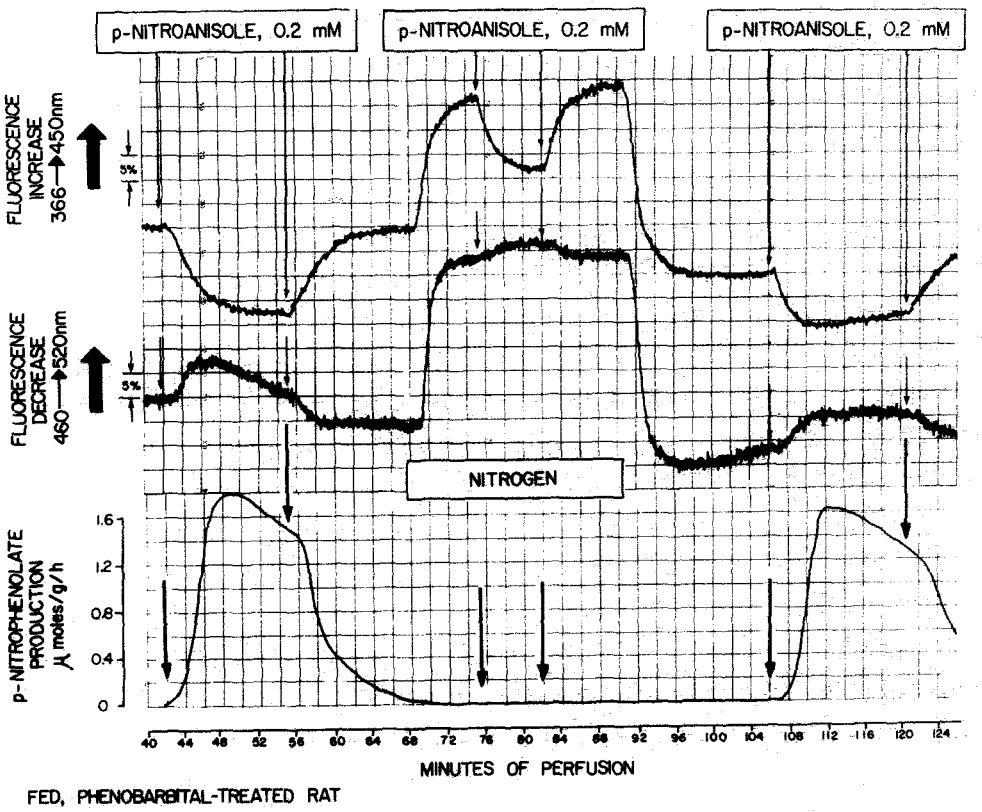

Fig. 1. Effect of *p*-nitroanisole *O*-demethylation on pyridine nucleotide and flavoprotein fluorescence in perfused livers from phenobarbital-treated female rats. Infusion of *p*-nitroanisole (0.2 mM) is indicated by the horizontal bars and arrows. Perfusate was saturated with an oxygen–carbon dioxide (95:5) mixture except during the period marked by the horizontal bar when it was saturated with a nitrogen–carbon dioxide (95:5) mixture. Surface fluorescence and *p*-nitrophenolate production were determined as decribed in Materials and Methods. One vertical scale division represents a 5 per cent change in fluorescence intensity.

Table 1. Effects of *p*-nitroanisole and hexobarbital on fluorescence of pyridine nucleotides and flavoproteins from the surface of the perfused liver *

Substrate	Fluorescence change (% of anoxia) Oxygen-saturated buffer	Nitrogen-saturated buffer	Difference
p-Nitroanisole (0.2 mM)			
Pyridine nucleotides	48 ± 10†	47 ± 11	1 ± 11
Flavoproteins	12 ± 6	3 ± 4	9 ± 5
Hexobarbital (0.25 mM)			
Pyridine nucleotides	31 ± 9	13 ± 3	18 ± 6
Flavoproteins	12 ± 3	5 ± 1	7 ± 2

* Changes in intensity of pyridine nucleotide or flavoprotein fluorescence resulting from *p*-nitroanisole or hexobarbital infusion into perfused rat livers were compared in four to six rats. Because the optical properties vary from liver to liver, data are expressed as per cent of fluorescence changes observed during a cycle of anoxia (maximal reduction due to infusion with nitrogen-saturated buffer). See Figs. 1 and 3.

† Mean ± S.E.M.

The mechanical and electronic details of the double fluorometer are described elsewhere [14]. The apparatus consists of a high intensity xenon lamp, a photomultiplier, a combination of primary and secondary filters mounted on a rapidly rotating disc (air driven), and necessary electronic components.

RESULTS

Surface fluorescence of pyridine nucleotides and flavoproteins during mixed-function oxidation. Infusion of *p*-nitroanisole into livers from fed, phenobarbital-treated rats resulted in the appearance of *p*-nitrophenolate in the effluent perfusion fluid (Fig. 1). Concomitantly, a large decrease in fluorescence excited at 366 nm and detected at 450 nm occurred. A similar decrease in surface fluorescence would be expected from oxidation of pyridine nucleotides. In addition, a small decrease in fluorescence excited at 460 nm and detected at 520 nm was observed, reflecting reduction of mitochondrial flavoproteins during metabolism of *p*-nitroanisole. However, when *p*-nitroanisole was infused in fluid saturated with 95% N_2:5% CO_2, a similar decrease in 366 → 450 nm fluorescence occurred

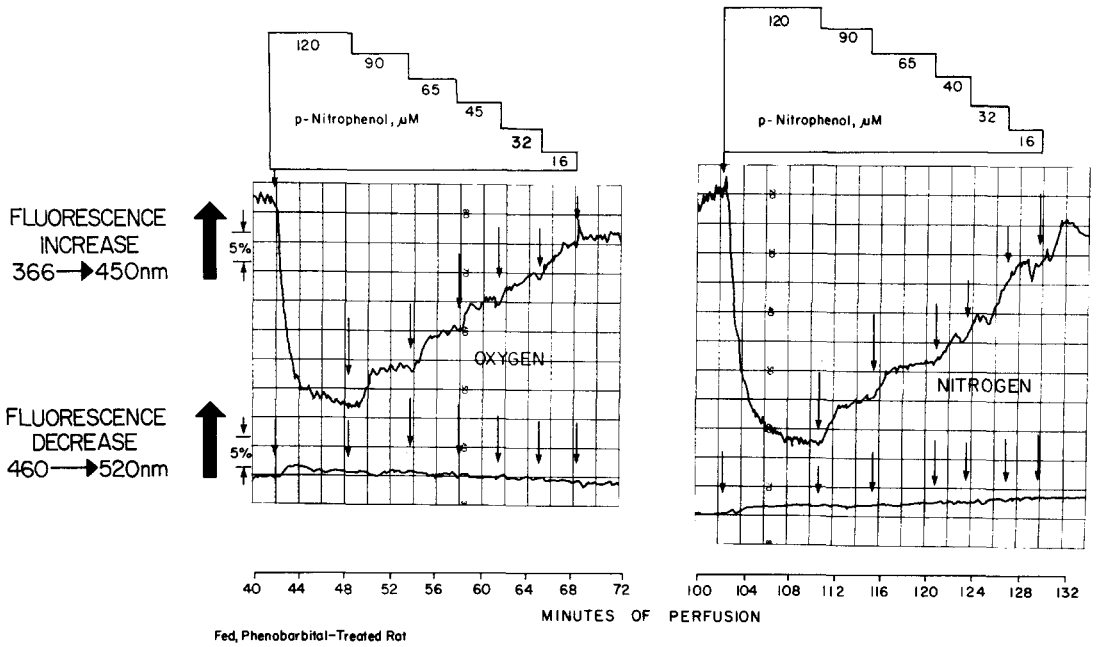

Fig. 2. Titration of pyridine nucleotide and flavoprotein fluorescence during perfusion of rat liver with oxygen- or nitrogen-saturated buffer. Perfusion fluid was saturated with an oxygen–carbon dioxide (95:5) (left panel) or a nitrogen–carbon dioxide (95:5) (right panel) mixture. Infusion of decreasing concentrations of *p*-nitrophenol is indicated by horizontal bars. Numbers indicate μmolar concentrations of *p*-nitrophenol. Conditions were the same as those described in Fig. 1.

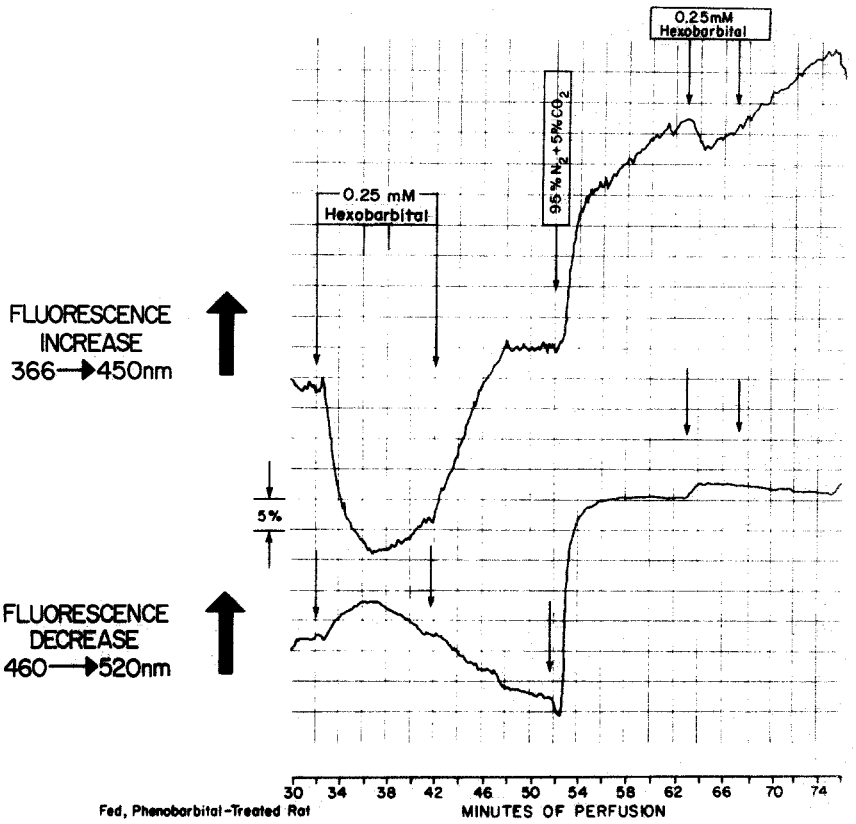

Fig. 3. Effect of hexobarbital on pyridine nucleotide and flavoprotein fluorescence in livers perfused with oxygen- or nitrogen-saturated buffers. Livers were perfused with oxygen-saturated buffer except after the period marked by the vertical bar, when nitrogen-saturated buffer was employed. Other conditions were the same as those described in Figs. 1 and 2.

(Fig. 1). Under these conditions, *p*-nitrophenolate production from mixed-function oxidation of *p*-nitroanisole was totally abolished due to lack of oxygen (Fig. 1). In contrast, changes in flavoprotein fluorescence were much smaller in nitrogen- than in oxygen-saturated buffer with *p*-nitroanisole. The changes in fluorescence (366 → 450) occurring with infusion of *p*-nitroanisole were quantitatively similar in oxygen- and nitrogen-saturated buffer (Table 1).

When *p*-nitrophenol, the major metabolite of mixed-function oxidation of *p*-nitroanisole, was infused into livers in the presence of oxygen- or nitrogen-saturated buffer, similar decreases in 366 → 450 nm fluorescence were observed (Fig. 2). Under both conditions, *p*-nitrophenol had little effect on flavoprotein fluorescence.

In contrast, hexobarbital produced a much greater (over 2-fold) decrease in 366 → 450 nm fluorescence (oxidation) when the liver was perfused with an oxygen-saturated rather than a nitrogen-saturated buffer (Fig. 3; Table 1). Small decreases in flavoprotein fluorescence occurred in both conditions (Table 1).

Aminopyrine, a model substrate for mixed-function oxidation, had no effect on surface fluorescence of pyridine nucleotides or flavoproteins (Fig. 4).

Because of the unexpected fluorescence decrease (366 → 450 nm) produced by *p*-nitroanisole and *p*-nitrophenol in nitrogen-saturated buffer, the optical absorption characteristics of these compounds were examined. *p*-Nitroanisole and *p*-nitrophenol absorb light strongly at 366 nm, indicating that they are capable of quenching 366 → 450 nm fluorescence (Table 2). In contrast, hexobarbital and aminopyrine absorbed light only minimally at wavelengths of interest.

Metyrapone (0.1 mM), a known inhibitor of mixed-function oxidation [17], inhibited *p*-nitrophenol formation from *p*-nitroanisole by 60–65 percent in an oxygen-saturated buffer (Fig. 5). Under these conditions, 366 → 450 nm fluorescence increased slightly. On the other hand, metyrapone infusion in the absence of *p*-nitroanisole produced a significant decrease in this fluorescence (19 per cent of maximal reduction during anoxia; Fig. 5, right panel). When *p*-nitroanisole was added subsequently in the presence of metyrapone, *p*-nitrophenol formation was inhibited approximately 80 per cent. Under these conditions an additional decrease of fluorescence excited at 366 nm was observed, which was the same magnitude as when *p*-nitroanisole was added alone. Similar effects on 366 → 450 nm fluorescence were observed from livers perfused with nitrogen-saturated buffer.

Effects of mixed-function oxidation on NAD⁺/NADH and NADP⁺/NADPH ratios. The influences of *p*-nitroanisole, hexobarbital and aminopyrine infusion on $NAD^+/NADH$ and $NADP^+/NADPH$ ratios are summarized in Table 3. The effects of 0.03 mM *p*-nitrophenol, which is the approximate concentration formed during *p*-nitroanisole *O*-demethylation by the

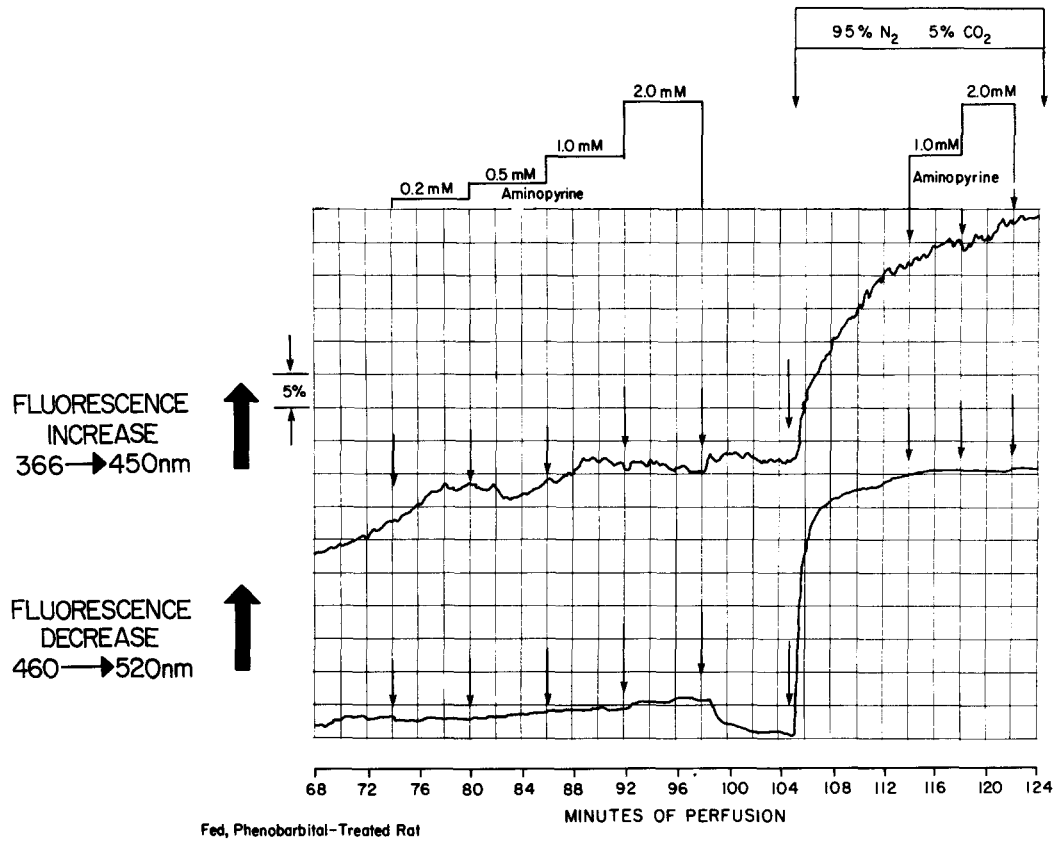

Fig. 4. Titration of pyridine nucleotide and flavoprotein fluorescence with aminopyrine in livers perfused with oxygen- or nitrogen-saturated buffers. Infusion of aminopyrine or nitrogen-saturated buffer is indicated by the horizontal bars. Conditions were the same as those described in Figs. 1 and 2.

perfused liver of a fed, phenobarbital-treated rat, are also included. Hexobarbital produced a statistically significant increase (oxidation) in the NADP⁺/NADPH ratio calculated from substrates assumed to be in near equilibrium with isocitrate dehydrogenase. A similar trend was observed in the data calculated from malic enzymes. In contrast, NADP⁺/NADPH ratios calculated from substrates in near equilibrium with isocitrate dehydrogenase were significantly lower during perfusion with *p*-nitroanisole (Table 3). In contrast, hexobarbital had no significant effect on the NAD⁺/NADH ratio. This calculation agrees with the oxidation of pyridine nucleotides detected from the surface of the perfused liver (Fig. 3; Table 1). NAD⁺/NADH ratios calculated from substrates assumed to be in equilibrium with lactate dehydrogenase tended to be lower in livers perfused with *p*-nitroanisole, *p*-nitrophenol and aminopyrine. The NAD⁺/NADH ratios calculated from substrates assumed to be in equilibrium with β-hydroxybutyrate dehydrogenase, an indicator of mitochondrial pyridine nucleotide redox state [18], decreased from 305 to 157 in livers perfused with *p*-nitroanisole. These data confirm the reduction of the mitochondrial NADH system detected by flavoprotein fluorescence in the presence of *p*-nitroanisole (Fig. 1; Table 1).

Table 2. Extinction coefficients of several mixed-function oxidation substrates at excitation and emission wavelengths for pyridine nucleotides and flavoproteins*

Drug	366 nm	ε · mM⁻¹ 436 nm	546 nm
p-Nitroanisole	1.2		
p-Nitrophenol	6.5	4.5	
Hexobarbital	0.013	0.01	0.008
Aminopyrine	1.4×10^{-4}	8.4×10^{-4}	7.2×10^{-4}

* Extinction coefficients were calculated from absorbance measurements of the compounds dissolved in Krebs–Henseleit bicarbonate buffer, pH 7.4, at 25° and light path length of 1.0 cm.

Since *p*-nitroanisole lowered the intracellular ATP/ADP ratio significantly, whereas hexobarbital and aminopyrine had no effect [3], we examined the action of *p*-nitrophenol on adenine nucleotides in livers of fed, phenobarbital-treated rats. Infusion of 0.03 mM *p*-nitrophenol for 6 min decreased the ATP/ADP ratio from 3.4 ± 0.3 (n = 5) to 2.7 ± 0.4 (n = 4).

DISCUSSION

Evaluation of changes in surface fluorescence during mixed-function oxidation. *p*-Nitroanisole and *p*-nitrophenol produced similar decreases of pyridine nucleotide fluorescence when infused into livers in buffer saturated with either oxygen or nitrogen (Figs. 1 and 2; Table 1). Although *p*-nitrophenol production from *p*-nitroanisole was totally inhibited in nitrogen-saturated

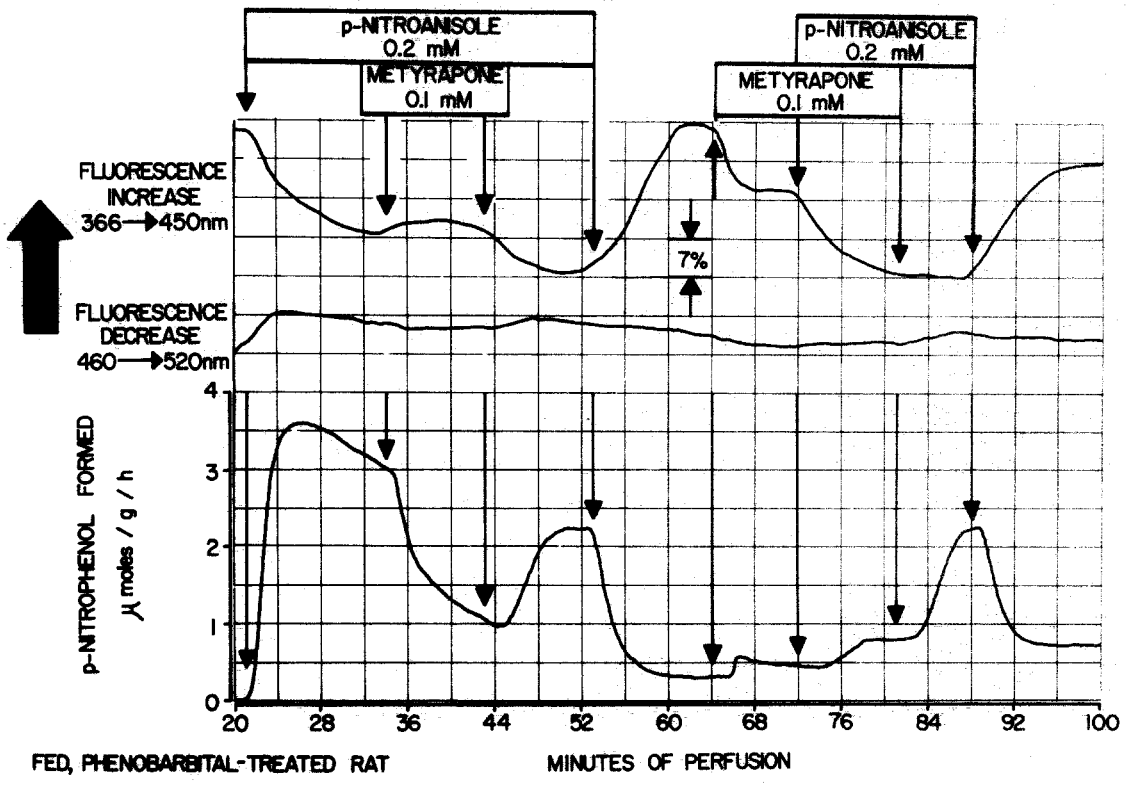

Fig. 5. Effects of metyrapone on *p*-nitrophenolate production from *p*-nitroanisole and flavoprotein and pyridine nucleotide fluorescence from the surface of a perfused rat liver. Conditions were the same as those described in Figs. 1 and 2.

buffer, the fluorescence decrease with *p*-nitroanisole (Fig. 1) or *p*-nitrophenol (Fig. 2) could result from nitroreductase activity [19]. However, a more likely explanation for this decrease in fluorescence is quenching by *p*-nitroanisole and *p*-nitrophenol.

Three arguments against the possibility that the decrease in fluorescence produced by *p*-nitroanisole and *p*-nitrophenol in oxygen-saturated buffer is due to NADPH oxidation come from experiments with an inhibitor of mixed-function oxidation, metyrapone [17]. First, metyrapone (0.1 mM) inhibited *p*-nitroanisole *O*-demethylation by 65 per cent, but failed to reverse the decrease in fluorescence due to *p*-nitroanisole (Fig. 5, left panel). If the fluorescence decrease was due to NADPH oxidation, metyrapone should have produced a large fluorescence increase. Second, when

Table 3. Calculated ratios of oxidized and reduced nicotinamide nucleotides in livers of phenobarbital-treated rats perfused with various drugs*

	$\frac{(NAD^+)}{NADH}$	$10^3 \times \frac{(NADP^+)}{(NADPH)}$	
	Lactate dehydrogenase	"Malic enzyme"	Isocitrate dehydrogenase
Control (19–24)	1118 ± 225	2.12 ± 0.35	1.96 ± 0.34
p-Nitroanisole (12)	870 ± 143	1.66 ± 0.30	0.92 ± 0.17†
p-Nitrophenol (5)	597 ± 242	2.39 ± 0.62	ND‡
Hexobarbital (10)	732 ± 103	2.69 ± 0.33	3.84 ± 0.56§
Aminopyrine (5)	735 ± 182	2.75 ± 0.47	ND

* The values ± S.E.M. (n) were calculated from the equilibrium constants for lactate dehydrogenase ($K = 1.11 \times 10^{-4}$ M), "malic" enzyme ($K = 3.44 \times 10^{-2}$ M), and isocitrate dehydrogenase ($K = 9.9 \times 10^{-7}$ M) and the measured values for lactate, pyruvate, malate, isocitrate and 2-oxoglutarate in freeze-clamped livers. Drugs were infused at the following concentrations: *p*-nitroanisole, 0.2 mM; hexobarbital, 0.1 mM; aminopyrine, 0.5 mM; and *p*-nitrophenol, 0.03 mM.

† $P < 0.05$.

‡ND = not determined.

§ $P < 0.01$.

metyrapone was added prior to *p*-nitroanisole, mixed-function oxidation was diminished by over 80 per cent, yet the decrease in fluorescence was as large as with *p*-nitroanisole alone (Fig. 5, left and right panels). Third, when metyrapone infusion was terminated, *p*-nitrophenolate production increased dramatically without a concomitant change in fluorescence. These data indicate that NADPH oxidation via mixed-function oxidation is not responsible for the decrease in fluorescence (366 → 450 nm) observed with *p*-nitroanisole (Fig. 1). This decrease can be ascribed to quenching because both *p*-nitroanisole and *p*-nitrophenol absorb light strongly at 366 nm, which is the excitation wavelength for pyridine nucleotide fluorescence (Refs. 14 and 15; Table 2). Quenching of pyridine nucleotide fluorescence was also observed when either *p*-nitroanisole or *p*-nitrophenol was added to solutions of NADH *in vitro* (L. A. Reinke and R. G. Thurman, unpublished observations). Because the fluorescence decrease in nitrogen is also due to quenching, any appreciable contribution of a nitroreductase to this decrease is unlikely.

Aminopyrine produced a small oxidation of NADPH (Table 3) which was not reflected in surface fluorescence. The best explanation for this observation is that formaldehyde generated from aminopyrine simultaneously caused a reduction of NAD^+ (Table 3) via NAD^+-dependent aldehyde and formate dehydrogenases. These data suggest that small changes in the calculated redox state of the $NADP^+$ system may not always be detected by surface fluorescence. Thus, measurements of surface fluorescence (366 → 450 nm) cannot be employed to evaluate redox changes accompanying mixed-function oxidation of all drug substrates since fluorescence quenching and reduction of NAD^+ preclude its use with *p*-nitroanisole and aminopyrine, respectively.

Limitations to evaluation of redox states employing $NADP^+$-dependent dehydrogenases. Limitations to obtaining valid estimates of intracellular redox states by calculated ratios stem both from low enzyme activity as well as from the presence of inhibitors which interfere with interactions between substrates, cofactors and enzymes. Good examples of the latter are ATP and ADP, which strongly inhibit $NADP^+$-dependent dehydrogenases [20]. The following observations suggest that valid esimates of redox states may not always be calculated from substrate measurements. Calculation of the $NADP^+$:NADPH ratios from substrates assumed to be in near equilibrium with different dehydrogenases do not always agree (Ref. 3; Table 3). Furthermore, during the mixed-function oxidation of *p*-nitroanisole, the ratio calculated from 6-phosphogluconate dehydrogenase [3] and isocitrate dehydrogenase (Table 3) changed, while that calculated from malic enzyme did not (Ref. 3; Table 3).

Since adenine nucleotides strongly inhibit $NADP^+$-dependent dehydrogenases, compounds which decrease the ATP/ADP ratio [e.g. *p*-nitroanisole [3] or *p*-nitrophenol (Results)] could stimulate NADPH generation through disinhibition of the dehydrogenases. The observed decrease in the $NADP^+$/NADPH ratio calculated from isocitrate dehydrogenase in livers perfused with *p*-nitroanisole may be explained by this mechanism. Changes in the activity of the dehydrogenases, therefore, may be responsible for changes in substrate and product levels for reasons other than equilibrium conditions.

On the other hand, hexobarbital, which did not affect intracellular ATP/ADP ratios, produced oxidation of pyridine nucleotides detected by surface fluorescence as well as by calculated $NADP^+$ ratios (Fig. 3; Tables 1 and 3). Furthermore, hexobarbital did not produce a fluorescence artifact because of its low extinction coefficients nor did it affect NAD^+ ratios under these conditions (Tables 2 and 3). Because surface fluorescence and calculated ratios both indicate oxidation of $NADP^+$ systems in the presence of hexobarbital, it is concluded that both methods indicate intracellular free $NADP^+$.

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REFERENCES

1. H. S. Mason, *Science* **125**, 1185 (1957).
2. H. Sies and M. Kandel, *Fedn Eur. Biochem. Soc. Lett.* **9**, 205 (1970).
3. F. C. Kauffman, R. K. Evans and R. G. Thurman, *Biochem. J.* **116**, 585 (1977).
4. H. A. Krebs and R. L. Veech, *Adv. Enzyme Regulat.* **7**, 397 (1969).
5. W. J. Marshall and A. E. M. McLean, *Biochem. Pharmac.* **18**, 153 (1969).
6. R. Scholz, W. Hansen and R. G. Thurman, *Eur. J. Biochem.* **38**, 64 (1973).
7. R. Scholz, in *Stoffweschel der perfundierten Leber* (Eds. W. Staib and R. Scholz), p. 24. Springer, Berlin (1968).
8. H. A. Krebs and K. Henseleit, *Hoppe-Seyler's Z. physiol. Chem.* **210**, 33 (1932).
9. R. G. Thurman, D. P. Marazzo, L. S. Jones and F. C. Kauffman, *J. Pharmac. exp. Ther.* **201**, 498 (1977).
10. A. Wollenberger, O. Ristace and G. Schoffa, *Pflügers Arch. ges. Physiol.* **270**, 399 (1960).
11. F. C. Kauffman, J. G. Brown, J. V. Passonneau and O. H. Lowry, *J. biol. Chem.* **244**, 3647 (1969).
12. O. H. Lowry and J. V. Passonneau, *A Flexible System of Enzymatic Analysis*, p. 147. Academic Press, New York (1972).
13. N. D. Goldberg, J. V. Passonneau and O. H. Lowry, *J. biol. Chem.* **241**, 3997 (1966).
14. B. Chance, V. Legallais, H. Sorge and N. Graham, *Analyt. Biochem.* **66**, 498 (1975).
15. R. Scholz, R. G. Thurman, J. R. Williamson, B. Chance and T. Bücher, *J. biol. Chem.* **244**, 2317 (1969).
16. J. R. Williamson, E. T. Browning and M. S. Olson, *Adv. Enzyme Regulat.* **6**, 67 (1968).
17. A. G. Hildebrandt, K. C. Leibman and R. W. Estabrook, *Biochem. biophys. Res. Commun.* **37**, 477 (1969).
18. T. Bücher, in *Pyridine Nucleotide Dependent Dehydrogenases* (Ed. H. Sund), p. 439. Springer, New York (1970).
19. P. H. Hernandes, P. Mazel and J. R. Gillette, *Biochem. Pharmac.* **16**, 1877 (1967).
20. F. C. Kauffman, R. K. Evans, L. A. Reinke and R. G. Thurman, *Biochem. J.* (in press).