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INTERACTION OF NITROMETHANE WITH REDUCED HEPATIC MICROSOMAL CYTOCHROME P-450

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Abstract—Nitromethane interacts with sodium dithionite reduced rabbit liver microsomes to generate a difference spectrum characterized by maxima at 388, 423, 454 and 520 nm and minima at 405, 436, 480 and 550 nm. Spectral binding constants (K_s) of 0.306 ± 0.082 mM (A_{max} = 0.032 ± 0.004), 0.178 ± 0.029 mM (A_{max} = 0.040 ± 0.002), and 1.168 ± 0.250 mM (A_{max} = 0.035 ± 0.006) were calculated for the 388, 423 and 454 nm peaks respectively. These difference spectra are qualitatively different from those previously reported for aromatic nitro compounds [L. A. Sternson and R. E. Gammans, *Drug Metab. Dispos.* **3**, 266 (1975)]. Interaction of nitromethane with microsomes from rabbits pretreated with phenobarbital produced absorbance maxima and minima within 2 nm of the controls. Interaction of nitromethane with reduced microsomes from 3-methyl-cholanthrene (3-MC)-pretreated animals produced ferrohemochromes in which maximal absorbance changes were shifted to maxima at 384, 422, 451 and 514 nm and minima at 407, 433, 473 and 552 nm. Peak-height ratios derived from difference spectra generated by the addition of 1 mM nitromethane to the sample cuvette were considerably different depending on whether the microsomes were obtained from control, phenobarbital- or 3-MC-pretreated rabbits and may indicate that phenobarbital, like 3-MC, induces qualitative changes in cytochrome P-450. Nitromethane apparently competes with carbon monoxide for a common binding site. Addition of nitromethane to CO-saturated microsomes reduced the magnitude of the 450 nm peak with a concomitant increase in the 423 nm peak of nitromethane. Similarly, addition of CO to reduced microsomes containing nitromethane caused reduction of the 423 nm peak of nitromethane with a corresponding increase of the 450 nm peak of CO. Nitromethane does not generate difference spectra with oxidized microsomes nor does it alter the K_s or A_{max} of aminopyrine, hexobarbital, aniline or zoxazolamine binding spectra. Nitromethane does inhibit the binding of the type II compound, nicotinamide. Addition of nitromethane to incubation flasks enhanced the metabolism of aniline while tending to inhibit the oxidative demethylation of ethylmorphine.

Interaction of many substrates with hepatic microsomal cytochrome P-450 is assumed to be essential for their oxidation. This interaction, resulting in the formation of substrate–P-450 complexes, often produces absorbance changes which are characteristic for the substrate. These changes are usually observed after interaction of the substrate with oxidized cytochrome P-450 with relatively few compounds interacting with microsomes in which the heme iron is in the reduced divalent state. Sternson and Gammans [1] reported that several aromatic nitro compounds interact with dithionite reduced rabbit liver microsomes to induce absorbance changes. Other compounds producing substrate binding spectra upon interaction with cytochrome P-450 include carbon monoxide [2], ethylisocyanide [3], metyrapone [4] and some structurally related pyridine derivatives [5]. We have investigated the interaction of nitromethane with rabbit liver microsomes and the results of this investigation are reported here.

METHODS

Microsomes were prepared from livers of male New Zealand white rabbits (1.5 to 3.0 kg) as previously described [6]. In specified experiments, enzyme induction was accomplished by the intraperitoneal injection of phenobarbital Na (80 mg/kg daily for 3 days) or 3-methylcholanthrene (20 mg/kg daily for 3 days) prior to sacrifice of the animals. The microsomal pellets obtained by differential centrifugation of the 9000 *g* supernatant of the 25% liver homogenate in 0.15 M KCl were routinely resuspended in 0.1 M phosphate buffer, pH 7.4. Washed microsomal pellets were stored at −25° for 1–3 days prior to use. Protein concentration of the resuspended pellets was determined by the method of Lowry *et al.* [7] using bovine serum albumin as protein standard. Cytochrome P-450 concentration was determined by the method of Omura and Sato [2].

Spectral changes associated with the addition of nitromethane to dithionite reduced microsomes (1.5 to 2.0 mg protein/ml) were measured under aerobic conditions with an Aminco DW-2 dual wavelength scanning spectrophotometer operated in the split beam mode. Nitromethane diluted with water was added in 1–40 μl volumes to establish concentrations of 0.05 to 2 mM. Spectral binding constants were calculated from measurements of absorbance differences between peaks and adjacent troughs.

When measuring the effect of nitromethane on the absorbance spectra of drug substrates, nitromethane was added to both cuvettes, a base line established and the drug substrate then added by microsyringe

in volumes of 1–40 μl (0.05 to 2.0 mM). The effect of nitromethane on the kinetics of hydroxylation *in vitro* of aniline and demethylation of ethylmorphine was measured by adding a 0.5 or 2.0 mM concentration of nitromethane to incubation flasks containing enzyme, cofactor and substrate (six concentrations, 0.125 to 3.0 mM). V_{max} and K_m values were calculated from 15-min incubations [8].

RESULTS

Like aromatic nitro compounds [1], nitromethane added to oxidized rabbit hepatic microsomes was incapable of generating difference spectra. Added to microsomes reduced with NADPH, a difference spectrum was observed which was characterized by a peak at 404 nm and a trough at 424 nm which intensified with time up to 40 min. With increased intensity, the peak absorbance shifted to 410 nm.

Nitromethane interacts with sodium dithionite reduced rabbit liver microsomes to generate difference spectra characterized by maxima at 388, 423, 454 and 520 nm and minima at 405, 436, 480 and 550 nm (Fig. 1). Spectral binding constants (K_s) for these interactions are presented in Table 1. Interaction of nitromethane with reduced microsomes from phenobarbital-pretreated rabbits produced absorbance difference spectra qualitatively similar to control microsomes with calculated spectral constants which are also shown in Table 1. Liver microsomes obtained from rabbits pretreated with 3-MC, when reduced with Na dithionite, induced formation of nitromethane–ferrohemochromes with maximal absorbance changes at 384, 422, 451 and 514 nm, with minima at 407, 433, 473 and 552 nm. The spectral binding constants (K_s) calculated for the three major absorbance peaks are different in control, phenobarbital- and 3-MC-pretreated groups indicating that there are three binding sites for the nitromethane—a high affinity site responsible for the 422–423 nm peak, an intermediate affinity site responsible for the 384–388 nm peak and a low affinity site responsible for the 451–454 nm peak.

The binding spectra observed upon addition of nitromethane to reduced microsomes are probably not due to reduction products of nitromethane, since methyl hydroxylamine has no absorbance peak at 454 nm and methyl amine does not produce a measurable difference spectrum when added to dithionite reduced microsomes.

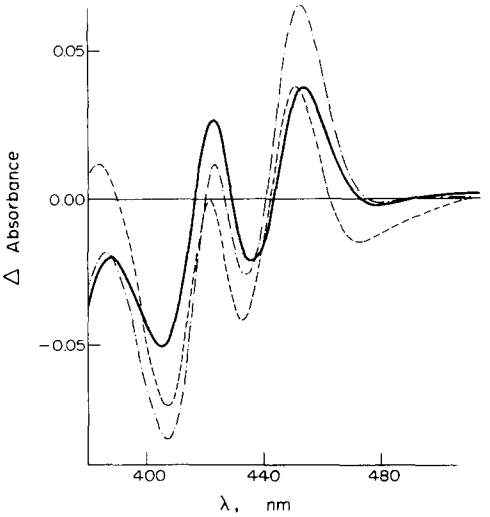

Fig. 1. Difference spectra generated by the interaction of nitromethane (1 mM) with dithionite reduced hepatic microsomes from control (——), 3-MC-pretreated (– – –) and phenobarbital-pretreated (—·—) rabbits.

Also Mansuy *et al.* [9] reported that the oxime having the next higher oxidation level after *N*-hydroxyamphetamine gave no difference spectrum with dithionite reduced rat liver microsomes nor does the related nitrosamine, dimethylnitrosamine (unpublished observations).

The difference spectra generated with nitromethane added to dithionite reduced microsomes are qualitatively different from those previously reported for aromatic nitro compounds [1]. The resemblance of nitromethane binding spectra to those of ethylisocyanide and the fact that 3-MC pretreatment induced qualitatively different spectral peak heights led us to investigate the influence of pH on these spectra. pH had negligible effect on the magnitude of spectral peaks in microsomes from (nontreated) control rabbits; however, increasing pH from 6.0 to 7.4 or 8.2 enhanced the absorbance at 384 nm of microsomes

Table 1. Apparent spectral dissociation constants (K_s) calculated for the interaction of nitromethane with reduced cytochrome P-450 from rabbit liver microsomes

Animal pretreatment	Absorbance maxima λ_{max}(nm)	K_s* (mM)	ΔA_{max}/ mg protein*
	388	0.306 ± 0.082	0.032 ± 0.004
	423	0.178 ± 0.029	0.040 ± 0.002
	454	1.168 ± 0.250	0.035 ± 0.006
Phenobarbital†	386	0.753 ± 0.153	0.065 ± 0.008
	423	0.182 ± 0.055	0.046 ± 0.006
	454	1.323 ± 0.272	0.102 ± 0.015
3-Methylcholanthrene‡	384	0.384 ± 0.073	0.044 ± 0.004
	422	0.205 ± 0.040	0.023 ± 0.002
	451	4.279 ± 1.755	0.064 ± 0.029

* Values are means ± S.E. for six determinations.

† Administered by intraperitoneal injection (80 mg/kg daily) for three days prior to sacrifice of animals.

‡ Administered by intraperitoneal injection (20 mg/kg daily) for three days prior to sacrifice of animals.

Table 2. Effect of peak-height ratios of nitromethane induced difference spectra from control, phenobarbital- and 3-MC-pretreated rabbits*

pH	Pretreatment	384/423 nm	423/454 nm
6.0	Control	0.30	2.83
6.0	Phenobarbital	0.35	2.62
6.0	3-MC	0.66	2.98
7.4	Control	0.43	2.43
7.4	Phenobarbital	2.21	0.80
7.4	3-MC	2.12	2.65
8.2	Control	0.57	2.54
8.2	Phenobarbital	2.04	0.59
8.2	3-MC	2.64	2.48

* Peak height measured from absorbance maximum to adjacent trough.

from both phenobarbital- and 3-MC-pretreated rabbits while decreasing absorbance at 422 nm in microsomes from phenobarbital induced rabbits. Also, at the more basic pH values, the absorbance change measured at 454 nm of microsomes from phenobarbital-pretreated rabbits was enhanced. Translating these absorbances to peak-height ratios (Table 2), it was possible to distinguish three different reactive forms of cytochrome P-450. Measuring peak heights at pH 7.4, the 384/423 peaks distinguish control from phenobarbital and 3-MC induced microsomes, whereas the 423/454 nm peak ratios distinguish phenobarbital induced microsomes from control and 3-MC induced microsomes.

The addition of nitromethane to CO-saturated or one-half saturated microsomes reduced the magnitude of the 450 nm peak with a concomitant increase in absorbance at 423 nm. Similarly, addition of CO to reduced microsomes containing nitromethane caused a reduction of the 423 nm peak of nitromethane with a corresponding increase of the 450 nm peak of CO. It appears that nitromethane competes with carbon monoxide for a common binding site. The rate of displacement of CO from the ferrohemochrome estimated as the decrease in magnitude of 450 nm absorbance after addition of nitromethane was found to be 0.775×10^{-3} and 0.1925×10^{-3} absorbance units/min at room temperature for control and 3-MC-pretreated rabbits respectively. The rate of CO displacement in microsomal suspensions in which the CO concentration was reduced to approximately one-half (microsomes diluted to proper protein concentration by adding equal volume of CO-saturated buffer) was 1.6×10^{-3} absorbance units/min.

The addition of nitromethane to microsomes of sample and reference cuvettes did not alter the spectral binding constants of hexobarbital, ethylmorphine, aminopyrine, zoxazolamine or aniline; however, it significantly inhibited the binding of the low-affinity type II substrate, nicotinamide (Table 3).

Neither nitromethane nor aromatic nitro compounds bind to oxidized microsomes; however, the latter were reported to markedly inhibit binding of type I substrates to cytochrome P-450 and to inhibit the oxidative metabolism of type II substrates in a system *in vitro* [10]. Nitromethane added to incubation flasks in a concentration of 0.5 or 2.0 mM significantly enhanced the V_{max} of aniline hydroxylase without affecting the K_m for this reaction. Similar concentrations of nitromethane added to incubation flasks containing the type I substrate, ethylmorphine, tended to reduce the V_{max} without significantly altering the K_m (Table 4).

DISCUSSION

The interaction of nitromethane with reduced hepatic microsomes appears to be both qualitatively and quantitatively different than that observed with aromatic nitro compounds. The difference spectra generated by interaction of aryl nitro compounds with reduced microsomes were characterized by maxima at 395–400, 420 and 514–520 nm with minima at 404, 444 and 550 nm; nitromethane produced difference spectra characterized by absorption maxima at 388, 423 and 454 nm. A broad absorption band was apparent with maximum at 520 nm and minimum at 550 nm similar to that observed with the aromatic compounds. Pretreatment of animals with phenobarbital did not alter the qualitative aspects of the nitromethane spectra nor did it induce significant quantitative changes (as measured by K_s or ΔA_{max}) in the 423 or 454 nm peaks. The spectral binding constant (K_s) for the 388 nm peak was significantly increased by phenobarbital pretreatment with a concomitant increase in ΔA_{max}. The magnitude of the difference spectrum, calculated as ΔA_{max}/mg of protein, may be considered to be a measure of the capacity of the binding site [1]. The significance of this increase in ΔA_{max} observed after phenobarbital pretreatment is not clear, but may suggest that the number of sites available for the interaction of nitromethane with the heme protein per unit weight of protein increases. However, the relative affinity of substrate for binding

Table 3. Effect of nitromethane (1 mM) on spectral binding constants of drug substances

Substrate	K_s (mM)	A_{max}*	In presence of nitromethane K_s	In presence of nitromethane A_{max}
Aniline	0.629 ± 0.068	0.031 ± 0.002	0.721 ± 0.070	0.028 ± 0.002
Hexobarbital	0.133 ± 0.014	0.031 ± 0.009	0.181 ± 0.023	0.034 ± 0.001
Zoxazolamine	0.507 ± 0.083	0.012 ± 0.001	0.469 ± 0.007	0.012 ± 0.001
Ethylmorphine	0.123 ± 0.007	0.0121 ± 0.0002	0.150 ± 0.018	0.0117 ± 0.0002
Aminopyrine	0.382 ± 0.052	0.0029 ± 0.0001	0.379 ± 0.023	0.0035 ± 0.0001
Nicotinamide	8.345 ± 1.879	0.064 ± 0.013	12.5†	0.018†

* A_{max} = absorbance difference/mg of microsomal protein.
† Estimated from Lineweaver–Burk plot.

Table 4. Effect of nitromethane on the kinetics of aniline and ethylmorphine metabolism by rabbit liver microsomes

Nitromethane concn (mM)	Aniline		Ethylmorphine	
	K_m (mM)	V_{max}*	K_m (mM)	V_{max}*
0	0.036 ± 0.009	9.6 ± 0.4	2.280 ± 0.077	97.4 ± 22.8
0.5	0.043 ± 0.010	14.7 ± 0.7†	0.921 ± 0.414	57.9 ± 13.6
2.0	0.030 ± 0.006	16.3 ± 0.5†	0.703 ± 0.258	46.4 ± 7.5

* Expressed as nmoles metabolized/mg of protein/hr.
† Significantly different from control, $P < 0.01$.

sites is often expressed as K_s, so the concomitant increase in K_s after phenobarbital pretreatment suggests that although the capacity of the binding site may increase, the affinity of nitromethane for the cytochrome decreases. Pretreatment of animals with 3-MC causes a slight alteration in the qualitative appearance of the nitromethane–ferrohemochrome complex, over that observed in untreated or phenobarbital-treated animals.

Hyposochromic shifts of all peaks were observed for the spectra obtained with 3-MC-treated preparations; the 388 nm peak is shifted to 384 nm, the 423 nm peak moved to 422 nm, the 454 nm band is shifted to 451 nm and the 520 nm peak to 514 nm. Relative nitromethane–ferrohemochrome spectral binding parameters (K_s and ΔA_{max}) for the peaks in the region of 385 and 423 nm are similar for cytochrome P-450 obtained from 3-MC-treated and untreated animals. The peak at 451 nm obtained with 3-MC cytochrome P-450 has a significantly higher K_s than the apparent corresponding peak at 454 nm obtained with untreated animals.

Addition of nitromethane to CO-saturated reduced microsomes reduced the magnitude of the 450 nm peak associated with CO-cytochrome interaction and resulted in a concomitant increase in the 423 nm peak of nitromethane. The rate of displacement of CO was approximately twice as fast when CO concentration of microsomes was reduced to approximately one-half saturation. Similarly, addition of CO to reduced microsomes containing nitromethane caused reduction in the magnitude of the 423 nm peak of nitromethane with a corresponding increase in the CO peak at 450 nm. These data suggest that nitromethane competes with carbon monoxide (CO) for common binding sites or there is at least a strong mutual dependency of the two ligands for reduced cytochrome P-450 binding sites.

Although nitromethane binds to reduced cytochrome P-450 with an affinity greater than that demonstrated for most aromatic nitro compounds [1] and appears to bind to the same site on cytochrome P-450 as CO, it affects drug-metabolizing reactions *in vitro* differently than aromatic nitro compounds or other ligands such as CO or metyrapone. Whereas CO nonselectively inhibits cytochrome P-450 linked oxidation, metyrapone inhibits several type II and type I substrate oxidations while aromatic nitro compounds only inhibit the metabolism of type II substrates. Nitromethane, although tending to reduce the V_{max} of the type I substrate, ethylmorphine, enhanced the V_{max} of aniline hydroxylation. These changes in velocity were not accompanied by significant changes in K_m for these reactions and, therefore, may be considered noncompetitive [11]. Metyrapone [11, 12] has also been shown to enhance type II substrate oxidation while generally inhibiting type I oxidation noncompetitively.

The ratio of peak heights from control, phenobarbital- and 3-MC-pretreated rabbits at various pH values are shown in Table 2. Peak-height ratios from control rabbit microsomes were only slightly altered by pH whereas a large change was noted in peak-height ratios of microsomes from phenobarbital- and 3-MC-pretreated rabbits when measured at different buffer pH. The absorbance ratios at pH 7.4 strongly suggest that nitromethane may be used to differentiate cytochrome P-450 derived from phenobarbital- or 3-MC-pretreated rabbits from those of nontreated rabbits. The 384/423 nm ratios of absorbance of microsomes from phenobarbital- or 3-MC-pretreated rabbits was about five times that of control microsomes, whereas the ratio of 423/454 absorbance calculated from microsomes obtained from control or 3-MC-pretreated rabbits was about three times that obtained from phenobarbital-pretreated rabbits. From these data it appears that pretreatment of rabbits with phenobarbital as well as 3-MC produced qualitative alterations in cytochrome P-450 which is manifest in an altered substrate binding spectrum when reacted with nitromethane.

Other evidence indicating qualitative changes in microsomal drug-metabolizing enzymes after phenobarbital pretreatment has been presented [13–17]. These include different patterns of drug and steroid hydroxylation [14, 17], different responses to specific antibody [15] and the induction of cytochrome P-450 of different molecular weights [13, 16].

In conclusion, nitromethane appears to be a useful reagent to sense qualitative variations among forms of cytochrome P-450 present in rabbit liver microsomes.

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