Effect of Aromatic Nitro Compounds on Oxidative Metabolism by Cytochrome P-450 Dependent Enzymes†

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The effect of aromatic nitro compounds on the oxidative metabolism of representative type I (hexobarbital and aminopyrene) and type II (aniline and zoxazolamine) substrates by cytochrome P-450 dependent liver enzymes was studied. Nitro compounds (nitrobenzene, *p*-nitrobenzoate, 2-nitrofluorene, and 2-nitronaphthalene) inhibited the oxidation of type II substrates by rabbit liver microsomal enzymes; however, they had no effect on the metabolism of the type I compounds. Inhibition of type II metabolism was characterized graphically as S,I-hyperbolic noncompetitive. The influence of aromatic nitro compounds on the interaction of type I and type II substrates with oxidized and reduced cytochrome P-450 was studied by difference spectroscopy. From Lineweaver–Burke plots, nitro compounds were shown to competitively interact with type II compounds for cytochrome P-450 binding sites. Nitro compounds completely prevented the appearance of a type I binding spectrum with either hexobarbital or aminopyrene even when the modifier was present at concentrations less than 10^{-8} *M*. Aromatic nitro compounds appear to therefore inhibit the metabolism of the type II substrates through a mixed mechanism of interaction with the microsomal drug-metabolizing enzymes.

We have recently reported[1] that aromatic nitro compounds interact with sodium dithionite reduced liver microsomes to generate difference spectra characterized by a maximum at 400 nm and minima at 440 and 528 nm. Although the nitro functional group can be reduced chemically under relatively mild conditions, the generated spectra are not due to an association of microsomes with some product of nitro reduction, since nitroso compounds, hydroxylamines, and amines failed to produce difference spectra under similar conditions.[1] Association was inhibited by carbon monoxide and metyrapone, suggesting that these ligands may be competing with nitro compounds for a common P-450 binding site or that an interrelationship between binding sites exists. It was further proposed that oxygen may affect the interaction of nitro compounds with reduced cytochrome P-450 thereby offering a possible explanation for the observed inhibitory effects of oxygen on microsomal nitro reductase.[2] If nitro compounds influence the coordination of O_2 with the hemoprotein, they may also affect reactions in which oxygen serves as an obligatory substrate, e.g., mixed function oxidations. Other ligands capable of forming ferrochemochromes with P-450, such as CO,[3] metyrapone[4] and its structural analogs,[5] and a benzphetamine metabolite,[6] inhibit mixed function oxidations. Accordingly, a study was initiated to determine the effect aromatic nitro compounds exert on the oxidation and spectral binding characteristics of type I (aminopyrine and hexobarbital) and type II (aniline and zoxazolamine) substrates. Gillette[7] has previously demonstrated that specific compounds which associate with cytochrome P-450 modify nitro reductase activity. Type II substrates (e.g., aniline) inhibited nitro metabolism while type I substrates exerted no effect or stimulated electron transfer to nitro moieties. The effect of nitro compounds on P-450 dependent oxidases is presented in this report.

Experimental Section

Microsomes were prepared from the livers of male New Zealand white rabbits (2–3 kg) as previously described.[8] Microsomal pellets were suspended in 0.15 *M* KCl solution containing phosphate buffer (0.10 *M*, pH 7.4) and recentrifuged at 105,000g to remove residual hemoglobin. Washed pellets were suspended in the ice-cold KCl–Tris–HCl solution; protein levels were determined by the Lowry[9] method (using bovine serum albumin as protein standard) and adjusted to appropriate concentrations with the buffer. Cytochrome P-450 levels were determined spectrophotometrically.[3]

All spectra were recorded under aerobic conditions with an Aminco DW-2 scanning spectrophotometer operated in the split beam mode.

Effect of Aromatic Nitro Compounds on the Binding of Aminopyrine, Aniline, Hexobarbital, and Zoxazolamine to Oxidized Microsomes. Washed microsomes (6 ml) (protein concentration 1.5 mg/ml) were divided between two cuvettes and a base line of equal light absorbance was recorded. Test substrates were added to the sample cuvette in 5–30 μl of buffer; an equal volume of buffer was added to the reference cuvette and a spectrum recorded. Aromatic nitro compounds which were dissolved in methanol were subsequently added (5–30 μl of a 15 m*M* solution) to both reference and sample cuvettes in equal concentration and spectra recorded.

Effect of Aminopyrine, Aniline, Hexobarbital, and Zoxazolamine on the Binding of Aromatic Nitro Compounds to Reduced Microsomes. Modification of the nitroferrohemochrome by type I and type II substrates was studied in a similar manner. Washed microsomes (1.5 mg of protein/ml) were reduced by the addition of sodium dithionite (3 mg), distributed between sample and reference cuvettes, and a base line of equal light intensity was recorded. Nitro compound was added to the sample cuvette in 5–30 μl of methanol; an equal volume of methanol was added to the reference cuvette and the spectrum recorded. Aniline, aminopyrine, hexobarbital, or zoxazolamine was subsequently added to both sample and reference cuvettes in equal concentration and the resultant spectra were recorded.

Metabolism Studies. Incubation mixtures used to follow the oxidative metabolism of aminopyrine, aniline, hexobarbital, and zoxazolamine contained enzyme equivalent to 250 mg of liver, NADP (2 μmol), glucose 6-phosphate (25 μmol), glucose-6-phosphate dehydrogenase (2 U), and $MgCl_2$ (30 mol) dissolved in 5 ml of 0.15 *M* KCl solution containing 0.10 *M* phosphate buffer (pH 7.4). Nitro compounds (final concentration in incubations, 0.1–2.0 m*M*) were deposited in incubation vessels in acetone solution before mixtures were constituted. The solvent was removed by evaporation and the nitro residue subsequently preincubated with the enzyme solution for 5 min at 37° before initiation of reactions. Reactions were initiated by the addition of cofactor. Zoxazolamine oxidase activity was monitored by established procedures;[10] aniline hydroxylase by the method of Imai;[11] hexobarbital oxidation according to the procedure outlined by Cooper and Brodie;[12] and aminopyrine *N*-demethylase was monitored by quantitating formaldehyde formed using the Nash assay.[13] In all cases, the presence of nitro compounds or their metabolites in incubation mixtures failed to effect the accuracy of these analytical procedures.

† Dedicated to the memory of Professor Edward E. Smissman, a fine teacher and good friend.

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Table I. Inhibition of Zoxazolamine Oxidase and Aniline Hydroxylase by Aromatic Nitro Compounds

Substrate	Inhibitor	K_{iI}[a-c]	K_{iS}[a-c]
Zoxazolamine[e]	Nitrobenzene	9.9 ± 0.3	9.1 ± 0.3
	p-Nitrobenzoate	4.3 ± 0.4	8.3 ± 0.2
	2-Nitronaphthalene	14.7 ± 0.2	5.3 ± 0.3
	2-Nitrofluorene[d]		
Aniline[f]	Nitrobenzene[d]		
	p-Nitrobenzoate	1.2 ± 0.1	9.3 ± 0.5
	2-Nitronaphthalene	3.3 ± 0.4	11.8 ± 0.6
	2-Nitrofluorene[d]		

[a] Values are means ± SEM × 10^{-4} *M*. [b] K_{iI} is defined as the concentration of inhibitor which produces a 50% increase in the vertical intercept of the double reciprocal plot of substrate vs. reaction velocity. [c] K_{iS} is defined as that concentration of inhibitor which produces a 50% increase in the slope of the double reciprocal plot of substrate vs. reaction velocity. [d] No significant inhibition observed at inhibitor concentration up to 5 × 10^{-3} *M*. [e] Zoxazolamine oxidase: K_m 16.6 ± 0.9 × 10^{-4} *M*; V_{max} 3.4 ± 1.2 nmol/mg of protein/hr. [f] Aniline hydroxylase: K_m 43.0 ± 0.8 × 10^{-4} *M*; V_{max} 4.9 ± 1.8 nmol/mg of protein/10 min.

Results

In order to determine the influence of aromatic nitro compounds on the oxidation of substrates by cytochrome P-450 dependent enzymes, the metabolism of type I (aminopyrine and hexobarbital) and type II (aniline and zoxazolamine) substrates was monitored in the absence and presence of representative nitro compounds, i.e., nitrobenzene, *p*-nitrobenzoate, 2-nitrofluorene, and 2-nitronaphthalene. Oxidation of the type II substrates was significantly inhibited by nitro compounds. Zoxazolamine metabolism was impaired by *p*-nitrobenzoate, nitrobenzene, and 1-nitronaphthalene but was not affected by 2-nitrofluorene. Aniline hydroxylase activity was reduced by 2-nitronaphthalene and *p*-nitrobenzoate; however, neither nitrobenzene nor 2-nitrofluorene effected oxidase activity. The basic type and extent of inhibition of oxidative metabolism caused by the nitro compounds was determined from reciprocal plots of reaction velocity vs. substrate concentration at different inhibitor levels (Figure 1, A).[14] Both slopes and intercepts changed as the concentration of the inhibitor increased and lines converged to the left of the vertical axis but did not show a single crossover point. Inhibition patterns were further analyzed by replotting slopes and intercepts vs. inhibitor concentration (Figure 1, B). Such replots were convex up with a horizontal asymptote at high nitro concentrations, and inhibition could therefore be classified as S,I-hyperbolic noncompetitive. The K_I for hyperbolic noncompetitive inhibition was determined by making plots of $(\text{slope}_i - \text{slope}_0)^{-1}$ or $(\text{intercept}_i - \text{intercept}_0)$ vs. $[I]^{-1}$, where slope_i is the slope at inhibitor concentration I and slope_0 is the slope when $I = 0$ (Figure 1, C).[14] The horizontal intercepts of such replots are defined as inhibition constants (K_{iS} for plots of slope; K_{iI} for plots of intercept). Inhibitory efficiency was therefore expressed as the concentration of nitro compound necessary to produce a 50% increase in the slope (K_{iS}) or intercept (K_{iI}) of reciprocal plots of reaction velocity vs. substrate concentration at different inhibitor levels (Table I). The absence of a single crossover point in the reciprocal plot is consistent with hyperbolic noncompetitive inhibition when $K_{i,denom}$'s are different in the slope and intercept terms.[15]

Metabolism rate of the type I substrates, hexobarbital and aminopyrine, was unaffected by the presence of any of the studied nitro hydrocarbons at concentration up to 1.5 m*M* in nitro compound.

The interrelationship between the binding of the substrates used in these metabolic studies and binding of nitro compounds to cytochrome P-450 was also investigated. The intensity of the classical type II spectra generated by the interaction of zoxazolamine or aniline with oxidized microsomes was reduced upon the addition of any of the nitro compounds (nitrobenzene, *p*-nitrobenzoate, 2-nitrofluorene, or 2-nitronaphthalene) in equal concentration to both sample and reference cuvettes (Table II). No qualitative alteration in the type II binding spectra was observed in the presence of modifier at concentrations from 0.1 to 1.5 m*M*. Reciprocal plots of absorbance changes (435–395 nm) vs. substrate (zoxazolamine or aniline) concentration at different concentrations of modifier (nitro compound) were constructed (Figure 2). The slopes of

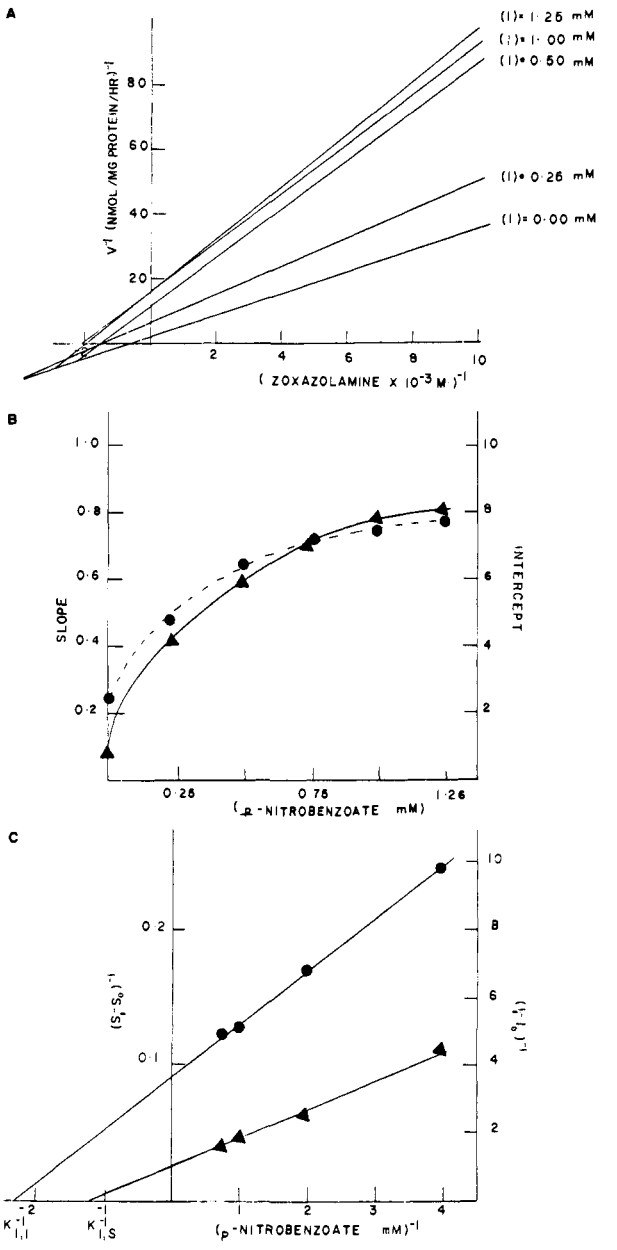

Figure 1. Graphic analysis of the inhibition of zoxazolamine oxidation by *p*-nitrobenzoate. Graph A illustrates the effect of various concentrations of *p*-nitrobenzoate (I) on the velocity of zoxazolamine oxidation. Replotting of the slopes (dashed line) or intercepts (solid line) vs. inhibitor concentration yielded the hyperbolas shown in graph B. Inhibitor constants were obtained from reciprocal plots of the change in slope $(S_i - S_0)$ (▲--▲) or the change in intercepts $(I_i - I_0)$ (● --●) vs. inhibitor (*p*-nitrobenzoate) concentration as shown in graph C, where slope_i is the slope at inhibitor concentration I and slope_0 is the slope when $I = 0$, and intercept_i and intercept_0 have similar meanings.

Table II. Effect of Aromatic Nitro Compounds on Spectral Constants of Zoxazolamine and Aniline in Washed Microsomes

Substrate	Modifier (concn)	K_s[a,b]	ΔA_{max}[a,c]
Zoxazolamine	None	0.83 ± 0.02	2.50 ± 0.23
	Nitrobenzene (1.0 m*M*)	2.32 ± 0.09	3.00 ± 0.32
	p-Nitrobenzoate (1.0 m*M*)	1.11 ± 0.06	2.52 ± 0.26
	2-Nitronaphthalene (0.1 m*M*)	1.46 ± 1.10	2.61 ± 0.32
	2-Nitrofluorene (0.1 m*M*)	1.32 ± 0.16	2.49 ± 0.26
Aniline	None	2.62 ± 1.10	2.85 ± 0.34
	Nitrobenzene (1.0 m*M*)	3.40 ± 0.16	2.50 ± 0.29
	p-Nitrobenzoate (1.0 m*M*)	3.40 ± 0.08	2.50 ± 0.32
	2-Nitronaphthalene (1.0 m*M*)	3.40 ± 0.10	2.50 ± 0.24
	2-Nitrofluorene (1.0 m*M*)	3.40 ± 0.06	2.50 ± 0.19

[a] Average values obtained from six-point double reciprocal plots using substrate concentrations from 10^{-4} to 1.5×10^{-3} *M* ($n = 8$). [b] Values are means ± SEM × 10^{-3} *M*. [c] Values are means ± SEM × 10^{-3}/nmol of P-450.

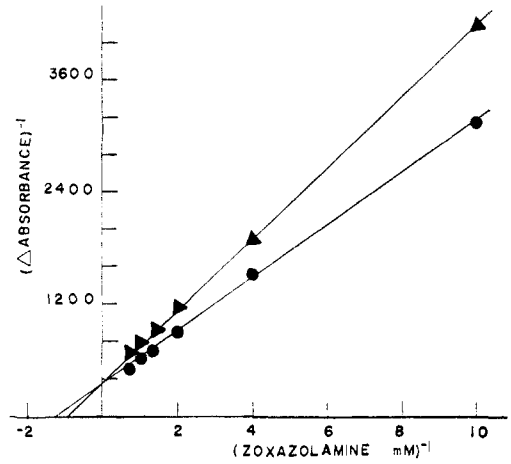

Figure 2. Effect of *p*-nitrobenzoate on the interaction of zoxazolamine with liver microsomes. Washed microsomes (6 ml) were divided between two cuvettes and a base line of equal light intensity was recorded. A family of difference spectra was generated by adding increasing amounts of zoxazolamine (final concentration, 0.1–1.5 m*M*) to the sample cuvette, adding an equal volume of buffer to the reference cuvette, and recording the spectra after each addition. The experiment was then repeated using microsomal suspensions which were 1 m*M* in *p*-nitrobenzoate. Lines shown in this figure were generated from reciprocal plots of the change in absorbance (435 minus 395 nm) vs. zoxazolamine concentration in the absence (●—●) and presence (▲—▲) of *p*-nitrobenzoate (1 m*M*).

these lines varied with modifier concentration but the intercept remained constant, suggesting that these interactions were competitive. Similar results were obtained when the effect of zoxazolamine or aniline on the difference spectra produced by the interaction of nitro compound with dithionite reduced microsomes was studied. Addition of zoxazolamine or aniline to both sample and reference cuvettes resulted in a decrease in the magnitude of the nitroferrohemochrome spectrum (produced by all four nitro hydrocarbons) without producing qualitative alterations in the spectra (Table III). Double reciprocal plots of absorbance changes (400–440 nm) vs. nitro concentration at various levels of modifier (zoxazolamine or aniline) again yielded curves typical of "competitive" binding, i.e., slopes varied with modifier concentration but intercepts remained unchanged (Figure 3).

Nitro compounds affected the binding of type I substrates (aminopyrine and hexobarbital) to oxidized microsomes in a much more dramatic manner than they influenced interaction of type II compounds with the cytochrome. The presence of any of the four nitro compounds in both sample and reference cuvettes (at concentrations as low as 10^{-8} *M*) completely prevented the appearance of characteristic type I spectral shifts upon the addition of aminopyrine or hexobarbital to the sample cuvette, even when these ligands were present at concentrations of 2m*M* (ca. 50 K_s). Addition of methanol alone to both cuvettes had no effect on the type I spectra produced by aminopyrene or hexobarbital.

Table III. Effect of Type I and Type II Substrates on the Spectral Constants of Aromatic Nitro Compounds in Dithionite Reduced Microsomes

Substrate	Modifier[a]	K_s[b,c]	ΔA_{max}[b,d]
Nitrobenzene	None	1.00 ± 0.06	4.54 ± 0.26
	Aniline	2.21 ± 0.14	4.49 ± 0.26
	Zoxazolamine	2.07 ± 0.18	4.46 ± 0.26
	Aminopyrine	1.07 ± 0.07	4.50 ± 0.23
	Hexobarbital	1.03 ± 0.10	5.34 ± 0.18[e]
p-Nitrobenzoate	None	1.18 ± 0.07	7.14 ± 0.26
	Aniline	1.98 ± 0.10	7.05 ± 0.20
	Zoxazolamine	2.06 ± 0.13	7.12 ± 0.16
	Aminopyrine	1.21 ± 0.06	7.09 ± 0.14
	Hexobarbital	1.12 ± 0.12	8.39 ± 0.34[e]
2-Nitronaphthalene	None	0.36 ± 0.05	5.56 ± 0.34
	Aniline	1.02 ± 0.10	5.49 ± 0.20
	Zoxazolamine	0.89 ± 0.10	5.51 ± 0.23
	Aminopyrine	0.35 ± 0.05	5.37 ± 0.28
	Hexobarbital	0.37 ± 0.05	6.46 ± 0.34
2-Nitrofluorene	None	1.08 ± 0.10	1.89 ± 0.24
	Aniline	2.00 ± 0.12	1.92 ± 0.24
	Zoxazolamine	1.96 ± 0.16	1.88 ± 0.34
	Aminopyrine	0.99 ± 0.13	1.93 ± 0.30
	Hexobarbital	1.09 ± 0.06	2.21 ± 0.19

[a] Modifiers were present in both sample and reference cuvettes at a concentration of 1 m*M*. [b] Values obtained from six-point double reciprocal plots using substrate concentrations from 10^{-4} to 1.5×10^{-3} *M*. [c] Values are means ± SEM × 10^{-3} *M*. [d] Values are means ± SEM × 10^{-3}/nmol of P-450. [e] Significantly increased at $p = 0.05$.

The effect of the presence of hexobarbital or aminopyrine in both sample and reference cuvettes on the difference spectra produced by the interaction of aromatic nitro compounds with reduced microsomes was also studied. At concentration as high as 2 m*M*, aminopyrine produced no alteration in the nitroferrohemochromes in which nitrobenzene, *p*-nitrobenzoate, 2-nitrofluorene, or 2-nitronaphthalene served as ligand (Table III). The presence of hexobarbital (1 m*M*) in both cuvettes, however, provided an approximate 20% increase in the binding propensity of nitrobenzene and *p*-nitrobenzoate to reduced microsomes as reflected by 20% increases in ΔA_{max} of the nitroferrohemochrome (Table III).

Discussion

The oxidative metabolism of type II substrates mediated by cytochrome P-450 dependent enzymes was significantly inhibited by aromatic nitro compounds while oxidation of type I substrates was not affected. Graphical analysis of the steady-state kinetics of aniline and zoxazolamine oxidation at varying concentrations of nitro compounds demonstrated that inhibition was S,I-hyperbolic noncompetitive, indicating that partial inactivation of P-450 occurs by interaction of the nitro substance with either the oxidized or reduced hemoprotein. Nonlinear noncom-

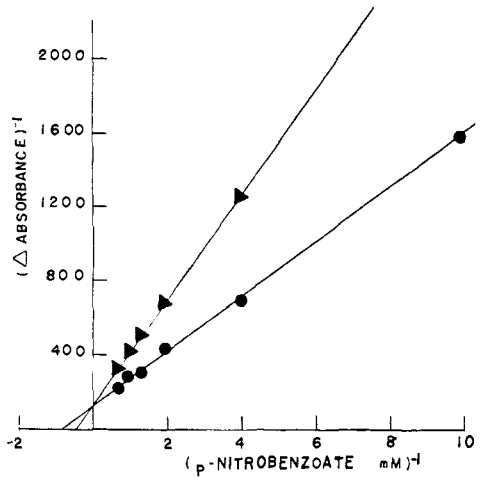

Figure 3. Effect of zoxazolamine on the spectral interaction of *p*-nitrobenzoate with dithionite reduced microsomes. Washed microsomes (6 ml) (1.5 mg of protein/ml) were reduced with 3 mg of sodium dithionite and divided between two cuvettes and a base line of equal light intensity was recorded. A series of difference spectra was recorded upon the addition of increasing amounts of a methanolic solution of *p*-nitrobenzoate (final concentration, 0.1–1.5 m*M*) to the sample cuvette, adding an equal volume of methanol to the reference cuvette and recording the spectra after the addition of each aliquot. The experiment was then repeated with microsomal suspensions which were 1 m*M* in zoxazolamine. Lines shown in this figure were generated from reciprocal plots of the change in absorbance (400 minus 440 nm) vs. *p*-nitrobenzoate concentration in the absence (●—●) and presence (▲--▲) of zoxazolamine (1 m*M*).

petitive inhibition is usually indicative of a mixed mechanism rather than a singular inhibitory pathway. Furthermore, it indicates that an incremental increase in nitro compound concentration does not produce increase in inhibition of oxidative metabolism. Curves generated by plotting slope or intercept vs. inhibitor (nitro) concentration (Figure 1, B) were hyperbolas, suggesting that at a higher concentration of nitro compound, inhibition approaches a limiting value. Although nitro compounds inhibit P-450 dependent processes and bind with reduced forms of cytochrome P-450, they do not mimic the action of such inhibitors as CO and metyrapone whose action apparently involves the combination with the reduced hemoprotein. CO and nitro compounds both produce difference spectra with only the reduced cytochrome; however, the spectral characteristics are quite different (CO, λ_{max} 450 nm; nitro compounds, λ_{max} 400 nm) and while CO inhibits all cytochrome P-450 catalyzed reactions, nitro compounds are much more selective. Metyrapone generates difference spectra with both oxidized and reduced P-450. The spectrum with reduced microsomes is again different than that formed with CO or nitro compound as ligand. Although both metyrapone and nitro compound selectively inhibit oxidative metabolism, metyrapone inhibits certain type I substrates while nitro compounds affect only type II substrates. Inhibition apparently results from interaction of nitro compounds at some site other than the classical type I or type II binding site, since (1) no difference spectra are generated by the combination of nitro compounds with oxidized microsomes and (2) nitro compounds do not competitively inhibit metabolism of type II substrates and do not affect the metabolism of type I compounds. However, nitro compounds do affect the binding of type I and II substrates to the oxidized hemoprotein. Results from spectral binding studies suggest that aromatic nitro compounds competitively interact with type II compounds for cytochrome P-450 binding sites. Reciprocal plots indicative of competitive binding were observed both when the effect of aromatic nitro compounds on type II spectra (with oxidized cytochrome) was studied and when the influence of type II compounds on the binding of nitro compounds to reduced microsomes was monitored. If nitro compounds form nonproductive complexes with cytochrome P-450, competitive binding would be observed if both ligands combine with the same enzyme form, although not necessarily at the same site. Such results are still consistent with results obtained from metabolism studies, since nonlinear noncompetitive inhibition patterns usually reflect a mixed mechanism of inhibition. From spectral studies, inhibition of metabolism appears to be in part due to nitro compounds directly effecting the binding of type II substrates to oxidized P-450 and type II compounds concomitantly interfering with the interaction of nitro compounds with the reduced hemoprotein. Selective inhibition by nitro compounds may result from a physical occlusion or conformational change in the enzyme at some alternative site interfering with metabolism of type II but not type I substrates.

Although the metabolism of type I substrates was not affected by the presence of nitro compounds, nitro-containing substances at concentrations as low as 10^{-8} *M* prevented the appearance of a type I spectrum with either hexobarbital or aminopyrine. No explanation is offered at this time to account for those observations. In other experiments, the presence of aminopyrine in both cuvettes was shown to have no effect on the spectra generated by the interaction of any of the nitro compounds with reduced microsomes. ΔA_{max} calculated for spectra generated by complexation of nitrobenzene or *p*-nitrobenzoate with reduced microsomes increased 20%, however, in the presence of hexobarbital. Increases in ΔA_{max} may reflect increases in binding affinity for the hemoprotein.[16] Therefore, hexobarbital apparently alters the binding characteristics of nitro compounds with reduced microsomes, while aminopyrine has no effect. These results are consistent with metabolism studies conducted by Gillette[7] who reported that aminopyrine has essentially no effect on nitro reductase, while hexobarbital stimulates reduction of nitro compounds by microsomal enzymes.

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