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NITRITE BINDING TO RABBIT LIVER MICROSOMES AND EFFECTS ON AMINOPYRINE DEMETHYLATION *

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Abstract—Sodium nitrite was examined for its ability to interact with liver microsomes from phenobarbital-treated rabbits. In dithionite- or NADPH-reduced microsomes, nitrite rapidly produced a difference spectrum with α, β, and Soret peaks at 586, 510, and 446 nm, respectively, with a large trough at 417 nm. The Soret peak diminished with time as the trough deepened and shifted to 429 nm. Concomitant with the development of the difference spectrum was a decrease in the ability of reduced cytochrome P-450 to bind carbon monoxide. When preincubated with NADPH-reduced microsomes under anaerobic conditions, nitrite behaved as a noncompetitive inhibitor of aminopyrine demethylase activity. Inhibition kinetics revealed two components with apparent inhibition constants of 0.2 and 31 mM nitrite. Maximum inhibition of aminopyrine demethylase by nitrite was obtained only after preincubation under anaerobic and reducing conditions. The rate and extent of inhibition were increased by decreasing the pH of the medium during preincubation. Inhibition of aminopyrine demethylation by nitrite does not appear to be mediated by oxidation effects of nitrite on lipids or essential sulfhydryl groups of cytochrome P-450. The evidence suggests that nitrite or a reduction product of nitrite, such as nitric oxide, binds tightly to reduced cytochrome P-450 to prevent carbon monoxide binding and inhibit aminopyrine demethylation activity.

The microsomal mixed-function oxidase system from mammalian liver consists of an NADPH-linked flavoprotein reductase and a family of similar but distinct species of cytochrome P-450. A number of compounds may bind to either oxidized or reduced cytochrome P-450 to produce characteristic spectral alterations. However, certain ligands may bind only to the reduced heme of cytochrome P-450 to form unique spectra. These ligands include carbon monoxide [1], isocyanides [2], amphetamines [3], and organic nitro compounds such as nitromethane [4], nitroalkanes [5], and aromatic nitro compounds [6]. The nitroalkanes have been shown to form 455 nm spectral complexes with reduced microsomes that can inhibit CO binding to cytochrome P-450. Nitromethane may also inhibit ethylmorphine demethylation in rabbit liver microsomes.

This paper describes the requirements for the binding of inorganic nitrite to rabbit liver microsomes and the subsequent inhibition of aminopyrine demethylase activity.

MATERIALS AND METHODS

Microsomal preparation. Male New Zealand white rabbits (2 kg) were maintained on Purina Rabbit Chow and water *ad lib.* 7 days prior to death. Rabbits received daily subcutaneous injections of 100 mg phenobarbital/kg body weight suspended in corn oil (100 mg/ml). Rabbits were fasted for 24hr, anesthetized with 50% carbon dioxide, and killed by incising the diaphragm. All tissue preparation was performed at 0–4°. The livers were excised, perfused with cold 0.15 M KCl, weighed, and brought to 5 vol. with 0.25 M sucrose, 50 mM Tris HCl (pH 7.6), and 1 mM ETDA. The tissue was homogenized in a Waring blender and the resulting homogenate centrifuged for 10 min at 23,500 *g*. The pellet was discarded and the supernatant fraction was centrifuged for 10 min at 23,500 *g*. The 23,500 *g* supernatant fraction was removed and centrifuged for 1 hr at 106,000 *g*. The resulting pellet was homogenized in 0.15 M KCl and centrifuged again at 106,000 *g* for 1 hr. This washed (microsomal fraction) was resuspended to about 40 mg protein/ml in 20% glycerol (v/v), 50 mM Tris–HCl (pH 7.6), and 1 mM ETDA and stored at −20°.

Microsomal absorption spectra. Spectrophotometric recordings were performed at 23–25° using either a Shimadzu model 200UV or an Aminco DW-2 spectrophotometer. The nitrite binding spectra were determined using liver microsomes (3 nmoles cytochrome P-450/ml) in 100 mM MOPS–KOH, pH 7.4.† Anaerobic studies of nitrite binding to isolated microsomes were performed by gently bubbling nitrogen gas through the microsomal suspension for 5 min. Samples were reduced where indicated with a few grains of sodium dithionite or an NADPH regenerating system consisting of 5 mM glucose 6-phosphate, 10mM $MgCl_2$, 1 mM $NADP^+$, and 2 units/ml of glucose 6-phosphate dehydrogenase. After obtaining a baseline, nitrite was added by depressing a plunger in the sample anaerobic cuvette.

The effects of nitrite on the CO binding spectrum were determined by reducing the microsomal suspension with a few grains of solid dithionite followed by addition of varying amounts of nitrite to both sample and reference cuvettes. The cuvettes were incubated at room temperature for 5 min after which CO was gently

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† Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; and tricine, *N*-Tris (hydroxymethyl) methylglycine.

bubbled through the sample cuvette for 60 sec. The resulting CO binding spectra were recorded after 5 min.

In determining the cytochrome P-450 concentration under various preincubation conditions, the microsomes were divided between sample and reference cuvettes, and a baseline was obtained. After the sample was bubbled gently with CO, both sample and reference were reduced with solid dithionite. The CO binding spectra were recorded 5 min later. The cytochrome P-450 concentration was determined using a mM extinction coefficient of 91 $cm^{-1}mM^{-1}$ for the 450 nm minus 490 nm absorption [1].

Assays. Aminopyrine demethylation assays were performed in a metabolic shaker (2 cycles/sec) at 37°. The assay system contained about 4 nmoles/ml of cytochrome P-450, 100 mM MOPS–KOH buffer (pH 7.5), 10 mM $MgCl_2$, 5 mM semicarbazide, and an NADPH regenerating system consisting of 5 mM glucose 6-phosphate, 1 mM $NADP^+$, and 2 units/ml of glucose 6-phosphate dehydrogenase in a final volume of 1 ml in a 20 mm bore test tube. When samples were preincubated with the regeneration system, additional 5 mM glucose 6-phosphate was added just before the reaction was started to replenish that lost due to glucose 6-phosphatase actvity. Anaerobic conditions for preincubations in the absence of substrate were achieved by placing the samples in a dessicator and alternately evacuating the chamber with a vacuum pump and refilling the chamber with nitrogen gas. After four cycles of this procedure, the dessicator was left under vacuum.

The reaction was started after 5 min of aerobic shaking by the addition of [dimethylamine-^{14}C]aminopyrine (10^5 cpm/μmole at the aminopyrine concentration indicated in the legends). Aliquots of 450 μl were taken at zero time and subsequent times thereafter, and immediately added to 3 ml chloroform. Unreacted substrate was extracted into the lower layer by vigorous vortex mixing for 3 min. Samples were centrifuged at 2000 *g* for 30 min and a 250 μl aliquot of the clear upper aqueous phase containing [^{14}C]-formaldehyde semicarbazone was added to 10 ml of aqueous counting scintillant and counted in a Beckman LS-200B liquid scintillation counter using [dimethylamine-^{14}C]aminopyrine standards of known specific activity. This procedure extracted about 99.8 per cent of the aminopyrine and no detectable formaldehyde (HCHO) into the chloroform phase. Although the formation of [^{14}C]HCHO was linear with time for over 30 min, the reaction was usually stopped at 12 min to determine initial rates of aminopyrine demethylation activity, which are expressed as nmoles HCHO formed/min·mg of protein.

NADPH cytochrome *c* oxidoreductase (EC 1.6.2.4) was assayed at 25° as follows. The sample cuvette contained about 100 μg of microsomal protein/ml and was incubated for 5 min in the presence of 100 mM potassium phosphate (pH 7.4), 3 mM NaN_3, 25 μM cytochrome *c*, 10 mM $MgCl_2$, 5 mM glucose 6-phosphate, and 2 units/ml of glucose 6-phosphate dehydrogenase. The reference cuvette contained 25 μM cytochrome *c* and 100 μg/ml of microsomal protein in potassium phosphate buffer. The reaction was started by the addition of various concentrations of $NADP^+$ to the sample cuvette and the increase in absorbance of cytochrome *c* was monitored at 550 nm. The mM extinction coefficient used for reduced minus oxidized cytochrome *c* was 21.1 $cm^{-1}mM^{-1}$ [7].

NADPH cytochrome P-450 oxidoreductase was determined at 25°. The sample and reference cuvettes contained about 5 mg of microsomal protein/ml in 100 mM potassium phosphate (pH 7.4). The sample was bubbled gently for 30 sec with CO, and the reaction was started by the addition of 1 mM NADPH to the sample cuvette. The increase in absorbance at 450 nm minus 490 nm was monitored for 15 sec (the reaction was linear for about 30 sec), and the results are expressed as nmoles cytochrome P-450 reduced/min·mg of protein, using an extinction coefficient of 91 cm^{-1} mM^{-1} for the 450 nm minus 490 nm absorption [1].

Protein was determined using the method of Lowry *et al.* [8] with bovine serum albumin used for the standard.

Chemicals. Cytochrome *c*, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (EC 1.1.1.49), $NADP^+$, phenobarbital, bovine serum albumin, tocopherol, 2-mercaptoethanol, dithiothreitol, dithioerythritol, and GSH were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Aminopyrine was from the Aldrich Chemical Co., Milwaukee, WI, U.S.A. ACS aqueous counting scintillant and [dimethylamine-^{14}C]aminopyrine were from Amersham/Searle, Arlington heights, IL, U.S.A. *N',N'*-diphenyl-*p*-phenylenediamine was obtained from the Eastman Kodak Co., Rochester, NY, U.S.A.

RESULTS

When nitrite was added to NADPH-reduced microsomes under anaerobic conditions, a difference spectrum appeared (Fig. 1, dashed line) with a peak at 446 nm and a trough at about 417 nm. This spectrum gradually developed to a final state (solid line) with α and β peaks at 586 and 510 nm, respectively, accompanied by a large Soret trough at 429 nm. With dithionite-reduced microsomes the final spectrum developed much more rapidly. After the final difference spectrum had developed, additional dithionite did not alter the spectrum. The concentration of nitrite required to produce a half-maximal absorbancy change at the final Soret minimum (spectral dissociation constant) was 37 μM. In oxidized microsomes nitrite did not produce absorbancy changes in the difference spectrum.

In order to determine whether nitrite was interacting specifically with cytochrome P-450, we tested the effect of preincubation with nitrite on CO binding to dithionite-reduced microsomes (Fig. 2). Increasing concentrations of nitrite inhibited both the rate and the extent of CO binding to dithionite or NADPH-reduced cytochrome P-450. Since O_2 is a cytochrome P-450 heme ligand as is CO, this result indicated that, under appropriate conditions, nitrite could act as a mixed-function oxidase inhibitor. Inhibition of aminopyrine demethylation required a preincubation of nitrite with microsomes under the same conditions necessary to produce a difference spectrum and inhibit CO binding (Table 1). Under these conditions of anaerobiosis in the presence of a reducing agent for cytochrome P-450, both the rate and extent of inhibition of aminopyrine demethylation

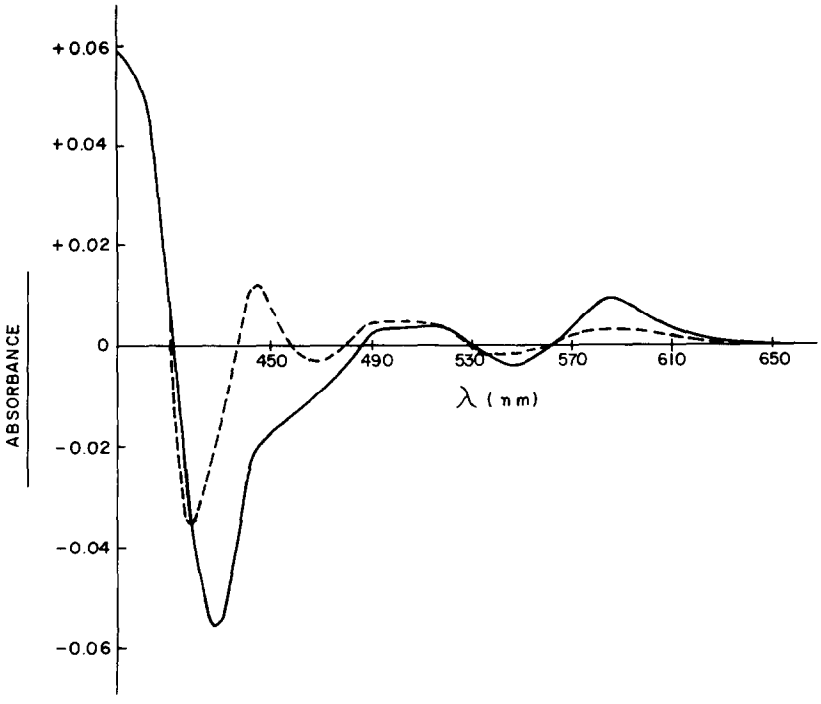

Fig. 1. Nitrite binding to anaerobic NADPH-reduced microsomes. Microsomes (3.2 nmoles cytochrome P-450/mg of protein) were suspended in 5 mM NADPH and 100 mM MOPS–KOH buffer (pH 7.5) at a final concentration of 3 nmoles cytochrome P-450/ml, and 3 ml were placed in sample and reference anaerobic cuvettes. The cuvettes were flushed with nitrogen for 10 min. After recording a baseline, 1 mM nitrite (final concentration) was added to the sample cuvette and the dashed spectrum (- - -) was recorded immediately. The solid spectrum(——) was recorded after 30 min. All spectral work was performed at 25°.

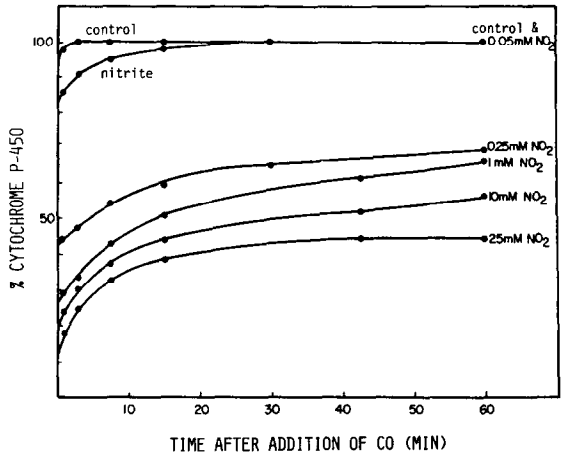

Fig. 2. Nitrite inhibition of carbon monoxide binding to cytochrome P-450. Microsomes (3.5 nmoles cytochrome P-450/mg of protein) were diluted to 1.5 nmoles cytochrome P-450/ml with 0.1 M MOPS–KOH (pH 7.5). The suspension was reduced with a few grains of solid dithionite. Various nitrite concentrations were added to the microsomal mixture which then was divided equally between the sample and reference cuvettes. After a 5-min preincubation period with nitrite at 25°, carbon monoxide was bubbled gently through the solution in the sample cuvette. The resulting (carbon monoxide + nitrite + dithionite-reduced microsomes) minus (nitrite + dithionite-reduced microsomes) spectra were recorded at the times indicated. The change in absorbance was measured at 450 nm minus 490 nm, and the results are expressed as a per cent of the original control cytochrome P-450 concentration (1.5 nmoles cytochrome P-450/ml).

by nitrite were enhanced by decreasing pH (Fig. 3). We have observed a maximum of 85 per cent inhibition in some experiments at pH 5.5, while at pH 8.5 the inhibition was barely detectable.

We next examined the possibility that nitrite was inhibiting mixed-function oxidase activity secondarily through an inhibition of the rate of reduction of cytochrome P-450 by the flavoprotein reductase. We found that nitrite up to 2 mM had no effect on the maximal velocity (140 ± 9 nmoles cytochrome *c* reduced/min·mg of microsomal protein) or K_m (6.0 ± 0.4 μM NADPH) of NADPH cytochrome *c* oxidoreductase. Furthermore, nitrite did not alter the ratio of nmoles NADPH oxidized/nmole of HCHO formed under any of our experimental conditions, nor did nitrite affect the maximal rate of NADPH cytochrome P-450 oxidoreductase (7.4 ± 0.5 nmoles cytochrome P-450 reduced/min·mg of microsomal protein). Therefore, it appears that the inhibition of aminopyrine demethylation by nitrite is not attributable to any effect on the transfer of electrons from NADPH to cytochrome P-450.

In order to determine whether inhibition of mixed-function oxidase activity by nitrite was at least partly due to the oxidizing effects of nitrite, rather than solely by binding to the cytochrome P-450 heme, a number of different reagents were included in the preincubation medium (Table 2). Nitrite has been shown to mediate lipid peroxidation [9] and produce free radicals [10]. Since hepatic mixed-function oxidase activity normally requires the presence of phospholipids [11], and lipid peroxidation has led to a loss in cytochrome P-450 [12], nitrite mediated lipid damage might be partly

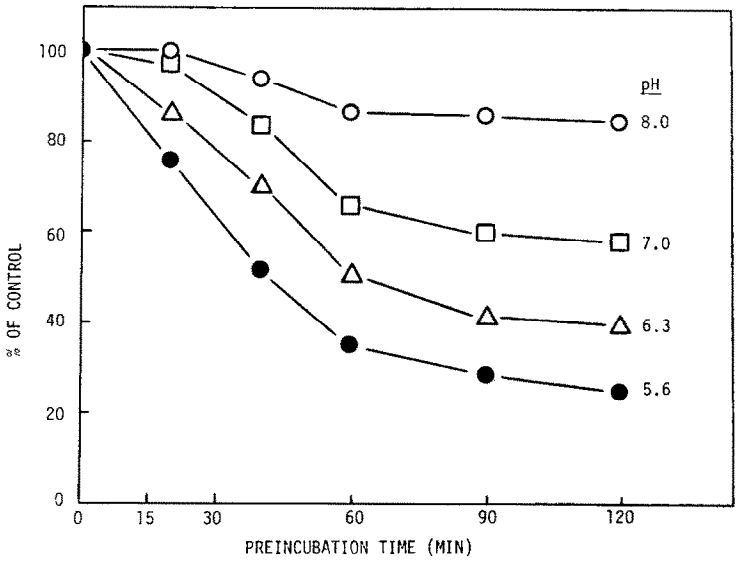

Fig. 3. Effect of pH during preincubation on the inhibition of aminopyrine demethylase by nitrite. Microsomes (3 nmoles cytochrome P-450/mg of protein) were preincubated under anaerobic conditions with 1 mM $NaNO_2$ and the NADPH regenerating system, as described in the legend to Table 1. The buffer consisted of 25 mM tricine + 25 mM MOPS + 25 mM maleic acid, adjusted to the pH indicated in the figure with HCl or NaOH. After preincubation for the indicated times, the samples were removed from the dessicators and 0.1 vol. of 1 M tricine + 1 M MOPS (pH 7.4) was added. The final pH of the samples during the assay of aminopyrine demethylation was 7.36–7.43. The reaction was started by adding 5 mM aminopyrine, and demethylation was assayed as described in Materials and Methods. The 100 per cent value at zero preincubation time was 4.2nmoles HCHO formed/min·mg of protein.

Table 1. Effect of preincubation conditions on aminopyrine demethylation *

	HCHO (nmoles formed/min·mg protein)		
	$-NaNO_2$	$+NaNO_2$	$\frac{+NaNO_2}{-NaNO_2}$
No preincubation	3.64 ± 0.21	3.73 ± 0.29	1.02
Anaerobic preincubation (no regenerating system)	3.52 + 0.14	3.41 ± 0.32	0.97
Anaerobic preincubation (with regenerating system)	3.54 ± 0.22	1.78 ± 0.15	0.50
Aerobic preincubation (with regenerating system)	3.08 ± 0.20	2.78 ± 0.16	0.90

* Microsomes (2.9 to 3.4 nmoles cytochrome P-450/mg of protein) were suspended in 10 mM $MgCl_2$ and 100 mM MOPS–KOH buffer (pH 7.5) at a final concentration of about 5 nmoles cytochrome P-450/ml. A regenerating system consisting of 5 mM glucose 6-phosphate, 1 mM $NADP^+$ and 1 unit/ml of glucose 6-phosphate dehydrogenase was added either before ("with regenerating system") or after ("no regenerating system") the 2-hr preincubation at 25°. Samples were rendered anaerobic by placing in a small dessicator and evacuating to about 10 mm Hg. The dessicator was refilled with nitrogen and the procedure repeated three times. Preincubations were performed in the absence or presence of 1 mM $NaNO_2$. After preincubation, the samples were aerated by vigorous shaking in a metabolic shaker at 37° for 5 min. The reaction was started by adding 5 mM aminopyrine, and demethylation was assayed as described in Materials and Methods. The results are expressed as the average of three determinations ± standard error of the mean.

Table 2. Effect of various agents on nitrite inhibition of aminopyrine demethylation*

	HCHO (nmoles/min·mg protein)		
	$-NaNO_2$	$+NaNO_2$	$\frac{+NaNO_2}{-NaNO_2}$
No additions	1.64 ± 0.14	0.87 ± 0.09	0.53
Dimethylsulfoxide (10 μl)	1.69 ± 0.17	0.84 ± 0.07	0.50
Tocopherol (1000 μg/ml)	1.74 ± 0.19	0.82 ± 0.07	0.47
DPPD (20 μg/ml)	1.39 ± 0.09	0.71 ± 0.04	0.51
ME (0.5 mM)	1.42 ± 0.13	0.75 ± 0.09	0.53
DTE (0.5 mM)	1.53 ± 0.08	0.72 ± 0.06	0.47
DTT (0.5 mM)	1.54 ± 0.10	0.79 ± 0.05	0.51
GSH (2 mM)	1.60 ± 0.14	0.82 ± 0.04	0.51
BSA (10 mg/ml)	1.72 ± 0.11	0.91 ± 0.10	0.53

* Microsomes (3.1 to 3.5 nmoles cytochrome P-450/mg of protein) were preincubated anaerobically as described in the legend to Table 1, in the presence of various agents listed above. Abbreviations used in the Table: DPPD, *N*, *N'*, diphenyl-*p*-phenylenediamine; ME, 2-mercaptoethanol; DTE, dithioerythritol; DTT, dithiothreitol; and BSA, bovine serum albumin. Tocopherol and DPPD were dissolved in dimethylsulfoxide immediately prior to use. After preincubation, the samples were treated as described in the legend to Table 1. The results are expressed as the average of three determinations ± standard error of the mean.

responsible for the inhibition of aminopyrine demethylase activity observed in the present study. However, diphenylphenylenediamine and tocopherol, antioxidants and quenchers of free radical propagation, were ineffective in protecting against nitrite inhibition. Also ineffective in protection were the sulfhydryl reagents 2-mercaptoethanol, dithiothreitol, dithioerythritol or reduced glutathione. These data suggest that nitrite is not mediating its inhibition either by oxidation or nitrosylation of a sulfhydryl group. Finally, bovine serum albumin did not protect against the inhibition of aminopyrine demethylation by nitrite, indicating that nonspecific activation of proteolytic enzyme activity is not part of the mechanism of inhibition.

We next tested the mode of inhibition by nitrite, to determine if increasing substrate concentration could restore aminopyrine demethylase activity. Nitrite was found to inhibit noncompetitively with respect to aminopyrine (Fig. 4A), exhibiting high and low affinity components. A Dixon plot of the data in Fig. 4A (shown in Fig. 4B) reveals apparent inhibition constants of 0.2 and 31 mM nitrite. The high affinity component is of primary interest in this study, since approximately 50 per cent inhibition of aminopyrine demethylase activity may be obtained with 1 mM nitrite.

DISCUSSION

The data presented in this paper suggest that nitrite may bind to the reduced heme of cytochrome P-450 and produce an inhibition of mixed-function oxidase activity. The reducing conditions required are similar to those necessary for the binding of other nitrogenous heme ligands, such as primary and secondary aliphatic nitroalkanes [5], nitromethane [4], and amphetamines [3].

The relationship between nitrite and CO binding in the present study is strikingly similar to that reported for aliphatic nitroalkane binding to rat liver microsomes [5]. When CO was bubbled into the sample prior to the addition of nitrite, nitrite failed to produce any absorbancy changes in dithionite-reduced microsomes. Conversely, in nitrite-treated dithionite-reduced microsomes, the CO-induced 450 nm peak was depressed. Not all the cytochrome P-450 was rendered insensitive to CO binding by nitrite (Fig. 2) which may reflect a population of cytochrome P-450 molecules that are less capable of binding or reacting with nitrite. Other ligands such as nitroalkanes [5], *N*-hydroxyamphetamine [15], diethylphenyl-phosphine [5], metyrapone [3], and octanethiol [16] have also been shown to bind between 10 per cent and 80 per cent of the total amount of reduced cytochrome P-450, suggesting heterogenous populations of cytochrome P-450 with respect to the binding of a number of other heme ligands.

The inhibition of aminopyrine metabolism by nitrite is probably due to the formation of nitric oxide from nitrite under the assay conditions. In aqueous solution, this reaction would be accelerated by anaerobic conditions and decreasing pH. However, it is more likely that NO formation is heme catalyzed, as indicated for the reaction of nitrite with myoglobin [17]. Anaerobiosis in the presence of a reducing agent accelerated the formation of nitrosylmyoglobin, presumably by a two-step procedure involving the oxidation of ferromyoglobin by nitrite to metmyoglobin plus NO which remained bound to the newly formed ferrimyoglobin. Similar mechanisms have been suggested for the effects of nitrite on other heme proteins, such as hemoglobin [18] and cytochrome *c* [19]. If nitrite inhibits aminopyrine demethylation by the heme catalyzed conversion of nitrite to NO, then nitrite must initially bind to

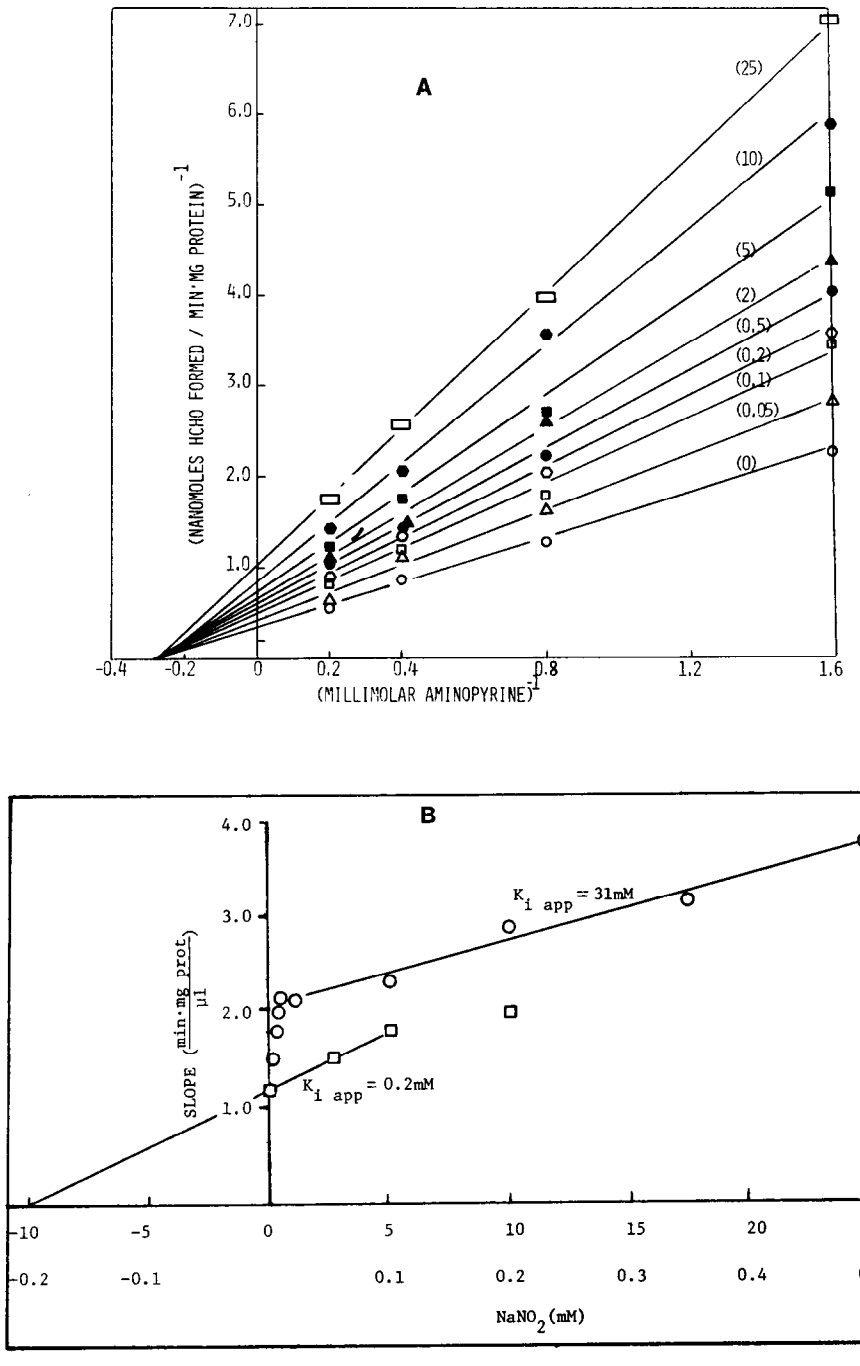

Fig. 4. Kinetics of aminopyrine demethylation in the presence of nitrite. Microsomes (3.0 to 3.5 nmoles cytochrome P-450/mg of protein) were preinubated under anaerobic conditions at 25°, as described in the legend to Table 1, with varying concentrations of $NaNO_2$ in the reaction mixture. Aminopyrine demethylation was initiated with 0.25, 0.5, 0.75 or 1 mM aminopyrine at constant specific radioactivity of 10^5 cpm/μmole. Panel A: reciprocal initial rates of aminopyrine demethylation are plotted against reciprocal aminopyrine concentrations for different $NaNO_2$ concentrations, according to Lineweaver and Burk [13] to determine V_{max} and K_m for aminopyrine. (mM $NaNO_2$ are in parentheses.) Panel B: the slopes of the double reciprocal plots of Fig. 4A are plotted against concentration of inhibitor, according to Dixon and Webb [14], to determine apparent K_i for the inhibitor.

the heme. Anaerobic preincubation conditions may be necessary for nitrite to bind if oxygen competes with nitrite for binding and has a higher affinity than nitrite for cytochrome P-450. If NO remains bound to ferrous or ferric cytochrome P-450, inhibition of mixed-function oxidations, may result from the formation of this high affinity complex, or from an actual destabilization of cytochrome P-450 as suggested by Ebel *et al.* [20]. These possibilities are currently being investigated.

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