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Evidence for General Catalysis and Formation of Nitrobenzene in the Oxidation of Phenylhydroxylamine in Aqueous Phosphate Buffer

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N-Phenylhydroxylamine is oxidized in aqueous phosphate buffer to nitrosobenzene, nitrobenzene, and azoxybenzene. Degradation is O_2 dependent and shows general catalysis by $H_2PO_4^-$ ($k_1 = 2.3\ M^{-2}\ sec^{-1}$) and PO_4^{-3} ($k_2 = 2.3 \times 10^5\ M^{-2}\ sec^{-1}$) or kinetically equivalent terms. Evidence is presented suggesting the intermediacy of a highly reactive species leading to these products.

Metabolic *N*- hydroxylation is postulated to be prerequisite for the carcinogenic activity of primary aryl amines (*1–5*). Although reports have described the chemical reactivity of the resulting arylhydroxylamine, these studies were carried out using analytical methodology incapable of simultaneously monitoring the reactant and product(s), and under conditions that are not physiologically relevant. The degradation of phenylhydroxylamine (PHA), the simplest representative of this chemical class, has been studied in nonaqueous solvents (*6–8*), at elevated temperatures (*9*), at pH extremes (*10, 11*), and with high concentrations of hydroxylamine ($\geq 10^{-2}\ M$) (*7*) under conditions where O_2 concentration was limiting. Its behavior was highly dependent on reaction conditions and resulted in the conversion of PHA to nitrosobenzene (PhNO), azoxybenzene (AzB), and/or *p*-aminophenol (PAP) (*12*), with differences observed in kinetic behavior and product distribution. Investigations of the photochemical (*13*) and electrochemical (*14, 15*) behavior of the compound have also been reported. In addition the fate of some arylhydroxylamines *in vivo* or in tissue preparations have been described in qualitative terms (*1–5*).

In the present report the chemical degradation of PHA, serving as a model for more potent amine-derived carcinogens, is described in oxygen-saturated, metal-free phosphate buffers (pH 6.8–7.4), to approximate a simplified (enzyme-free metal-free, etc.) biological environment.

RESULTS AND DISCUSSION

Phenylhydroxylamine degrades in aqueous (O_2-saturated) phosphate buffer

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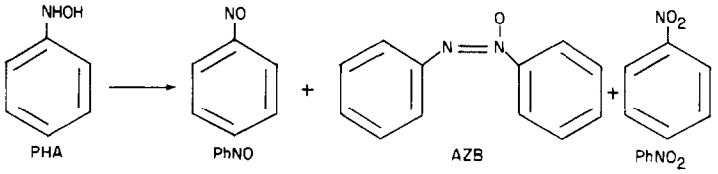
solution (pH 6.8–7.4) at 25°C to yield PhNO, AzB, and nitrobenzene ($PhNO_2$) (Eq. [1]).



[1]

$PhNO_2$ has not been previously detected as a product of PHA oxidation and appears from this study to form from a reactive intermediate generated during oxidation. The identity of the products was confirmed based on their chromatographic behavior (hplc), i.e., having retention volumes identical to authentic samples of these three compounds on RP-18 and μ-Bondapak NH_2 columns using a range of methanol : water and 2-propanol : water mobile phases. In addition, electron impact mass spectra and elemental analysis of each compound corresponding to the hplc peaks assumed to be PhNO, $PhNO_2$, or AzB were consistent with the analytical data for the authentic compounds.

An hplc method was developed to permit specific and simultaneous monitoring of substrate and all products during reaction. Oxidation is first order in PHA for at least five half-lives and is oxygen dependent. A plot of log k_{obs} vs log $[O_2]$ was linear (Fig. 1) with a slope of 0.93, demonstrating the first-order dependency of the reaction on O_2 concentration. $PhNO_2$ formation also exhibits strictly first-order

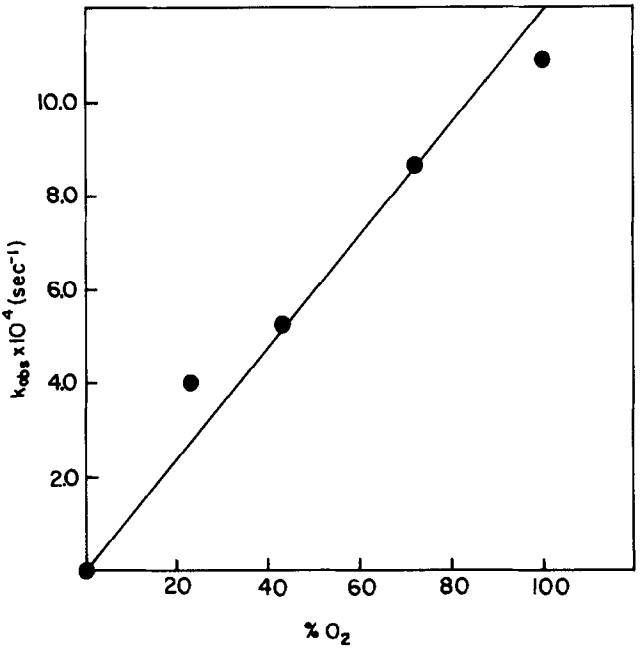


FIG. 1. Plot of k_{obs} vs percentage O_2 in phosphate buffer (pH 7.4; 0.01 M, μ = 0.5, 25°C) for the oxidation of phenylhydroxylamine, determined at 0, 23, 42, 72, and 100% O_2.

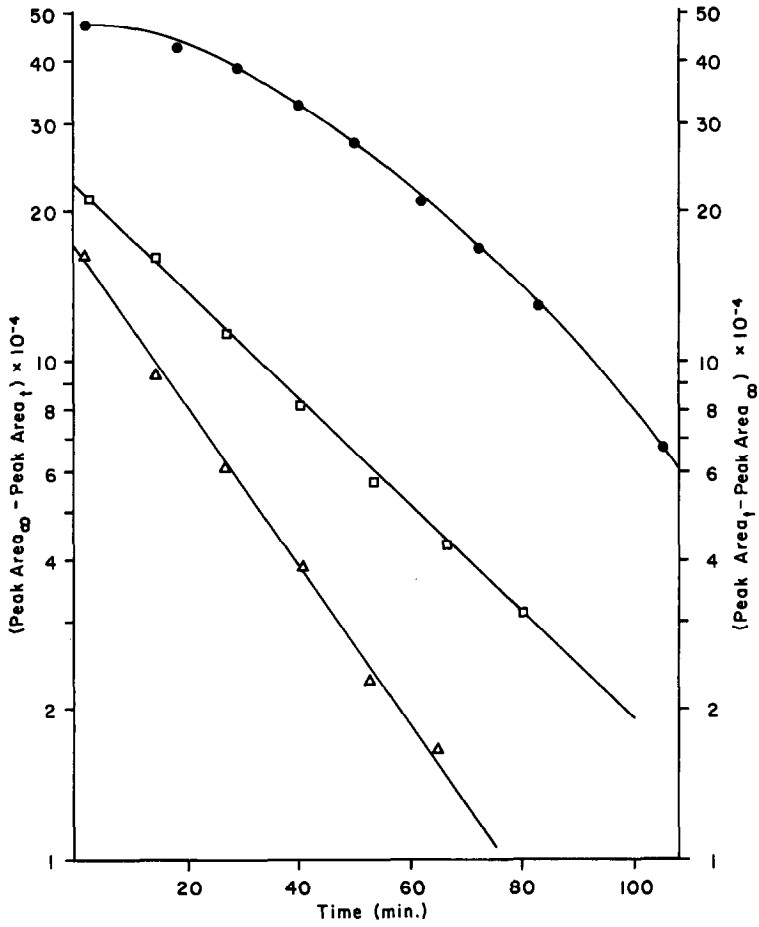


FIG. 2. Distribution profile of PHA (△) disappearance (calculated as peak area$_t$ − peak area$_\infty$, where peak area$_\infty$ = 0) and AzB (●) and $PhNO_2$ (□) appearance (calculated as peak area$_\infty$ − peak area$_t$) in 0.01 *M* phosphate buffer (ph 7.4; μ = 0.5, $NaClO_4$) at 25°C.

kinetics; however, the value of the rate constants for $PhNO_2$ formation are always ca. 80% of the value of k_{obs} for PHA disappearance. A typical profile of the species distribution for PHA oxidation as a function of time is shown in Fig. 2.

Oxidation is subject to general catalysis by phosphate buffer. The dependency of k_{obs} on total phosphate concentration (P_t) at the four pH's of this study is shown in Fig. 3. A kinetic expression for k_{obs} in accord with the experimental data is given by

$$k_{obs} = (k_1[H_2PO_4^-] + k_2[PO_4^{3-}] + k_{ox})[O_2], \quad [2]$$

where k_{ox} represents a buffer-independent rate constant. Kinetically equivalent terms for $[H_2PO_4^-]$ and $[PO_4^{3-}]$ can be written which at this time cannot be eliminated from consideration. Upon rearranging Eq. [2] and substituting for $[H_2PO_4^-]$ and $[PO_4^{3-}]$ in terms of P_t,

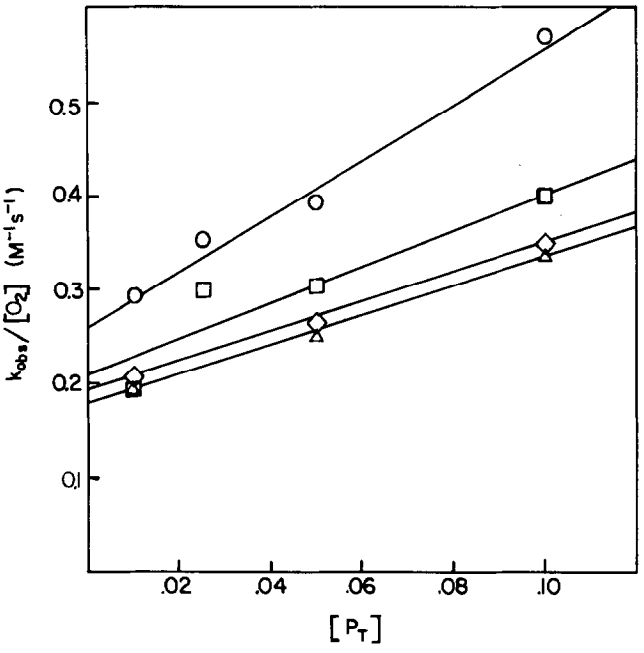


FIG. 3. Plots of $k_{obs}/[O_2]$ vs total phosphate concentration for the general catalyzed oxidation of phenylhydroxylamine (μ = 0.5, 25°C) at pH 6.75 (△), 7.04 (◇), 7.25 (□), and 7.40 (○). Each point represents the average of triplicate determinations.

$$k_{obs}/[O_2] = \left\{\frac{k_1 a_H^2 + k_2 K_{a_2} K_{a_3}}{a_H^2 + K_{a_2} a_H + K_{a_2} K_{a_3}}\right\} P_t + k_{ox} \qquad [3]$$

is obtained where K_{a_2} = acid dissociation constant for $H_2PO_4^-$ ($10^{-6.5}$) (*16*), K_{a_3} = acid dissociation constant for HPO_4^{2-} ($10^{-12.3}$) (*17*), and a_H = activity of hydrogen ion as measured by the glass electrode. The bracketed term (Eq. [3]) and the value of k_{ox} at each pH are thus obtained graphically from the slope and intercept, respectively, of a plot of $k_{obs}/[O_2]$ vs P_t (Fig. 3). Over the pH range studied (6.75–7.40), $a_H^2 + K_{a_2} a_H \gg K_{a_2} K_{a_3}$, thus simplifying the mathematical expression for the slope so that a secondary plot of slope/a_H/($K_{a_2} + a_H$) vs a_H^{-2} (Fig. 4) is linear with an intercept of k_1 (2.3 M^{-2} sec^{-1} third-order rate constant for catalysis by $H_2PO_4^-$) and a slope of $k_2 K_{a_2} K_{a_3}$ (from which k_2, the third-order rate constant for catalysis by PO_4^{3-} was calculated to be 2.3 × 10^5 M^{-2} sec^{-1}). Simplex fit of the data suggests that a term in $[HPO_4^{2-}]$ or a kinetically equivalent expression does not contribute to the overall rate expression.

Although the value of k_{ox} decreased with decreasing pH, the linear plot of k_{ox} vs a_H^{-1} gave a non-zero intercept. Including a pH-independent term (k_0), k_{ox} can be expressed by

$$k_{ox} = k_{HO} K_w / a_H + k_0, \qquad [4]$$

where k_{HO} is the hydroxide-dependent term (k_{HO} = 2.3 × 10^5 M^{-2} sec^{-1}) and k_0 is

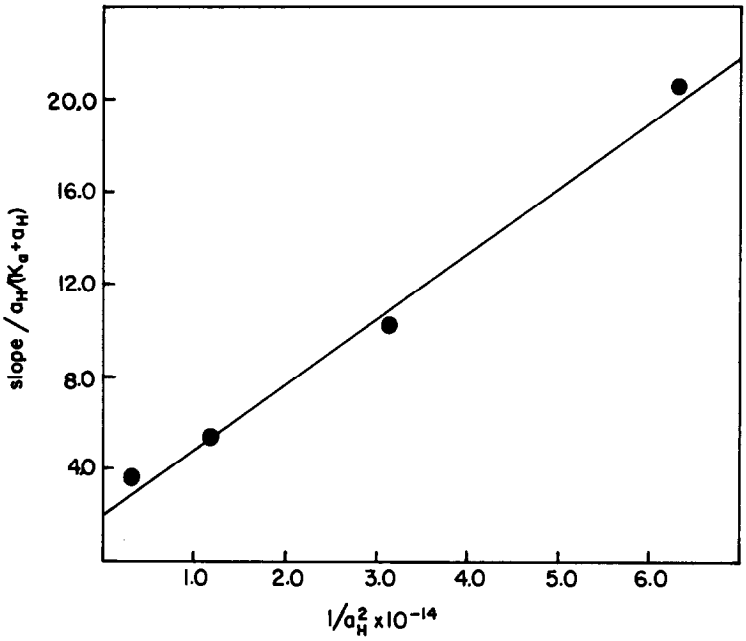


FIG. 4. Secondary plot of slope/$a_H(K_a + a_H)^{-1}$ vs a_H^{-2} for the oxidation of phenylhydroxylamine in phosphate buffer. The theoretical line was computer generated by a least-squares program.

the spontaneous term ($k_0 = 1.5 \times 10^{-1} M^{-1}$ sec^{-1}). Due to the narrow pH range studied, k_{HO} and k_0 can only be approximated. Outside of this pH range, additional products are formed and the description of chemical behavior is further complicated.

Product distributions were determined at four pH's at constant P_t (Table 1). $PhNO_2$ and AzB are stable under the experimental conditions but the high vapor pressure of PhNO caused difficulties in its quantitation. From control experiments, incubation of PhNO under the experimental conditions resulted in a ca. 20% loss with the appearance of no new products, substantiating the hypothesis of the partial volatilization of PhNO and subsequent loss via the gassing system. Mass balance became more acceptable under conditions where rates were faster (see Table 1). The product distribution shows no correlation in any simple manner with the degree to which the kinetic terms of Eq. [2] contribute to the rate of disappearance of PHA. The data do show, however, that catalysis by phosphate buffer is not associated with catalysis of the condensation reaction since as the percentage of the pathway through the phosphate terms increase, the yield of AzB decreases. Clearly phosphate catalyzes the oxidation reaction—either affecting the initial step or the breakdown of an intermediate involved in a rate-determining step.

Whereas NOB is formed by direct action of O_2 on PHA (*7*) and AzB forms from condensation of PHA with PhNO (*18*), the nitrogenous precursor of $PhNO_2$ and the oxidizing agent responsible for $PhNO_2$ formation must be elucidated. $PhNO_2$ does not arise from simple O_2-mediated oxidation of PhNO or AzB since both

TABLE 1

THE PERCENTAGE WHICH EACH KINETIC PATHWAY[a] CONTRIBUTES TO k_{obs} AND THE RELATIVE PERCENTAGE OF PRODUCTS FORMED FROM PHA UNDER THE SAME EXPERIMENTAL CONDITIONS[b]

	Percentage						
pH	$k_1[H_2PO_4^-]$	$k_2[PO_4^{3-}]$	$k_{OH}[^-OH]$	k_0	$PhNO_2$	AzB	PhNO[c]
6.31	46	2	2	50	12	20	68(46)
6.83	27	16	5	52	12	16	72(54)
7.24	10	39	10	40	12	10.5	77.5(56)
7.84	1	68	16	15	10.5	5	84.5(69)

[a] k_{ox} has been separated into its two components (i.e., k_{OH} and k_0) as described by Eq. [4].

[b] 0.91 MP_i, 25°C, μ = 0.5, $NaClO_4$. All values represent the average of triplicate determinations.

[c] The assumption is made that the difference in observed yield and 100% is due to mechanical loss of PhNO (see text). Therefore, the % yield of PhNO in the above table has been increased to provide a 100% total yield of products at all pH's. The numbers in parentheses are the original yields of PhNO before correction.

compounds are stable in O_2-saturated buffer. Although no simple relationship exists between the product ratios (obtained as a function of pH) and the kinetic terms of Eq. [2] (Table 1), the yield of $PhNO_2$ remains constant over the pH range studied. Thus, it appears that $PhNO_2$ forms by a pathway independent of PhNO or AzB. This presumption is supported by the first-order behavior observed for $PhNO_2$ formation. A lag phase would be anticipated if PhNO or AzB were precursors to $PhNO_2$ formed in a rate-determining step (RDS); whereas, if the final oxidation was fast relative to the RDS, a lag phase would not be observed, but then a correlation between [PhNO] (or [AzB]) and [$PhNO_2$] would be anticipated. It thus appears that $PhNO_2$ forms directly from PHA.

Bamberger (*12*) has previously shown that the conversion of PHA to PhNO proceeds with the generation of hydrogen peroxide. The involvement of H_2O_2 in formation of $PhNO_2$ was investigated. NOB is quantitatively converted to $PhNO_2$ with the value of the rate constant for NOB disappearance ($k_{H_2O_2} = 2.8 \times 10^{-5}$ M^{-1} sec^{-1}) equal to that rate constant for $PhNO_2$ formation. However, assuming the maximum concentration of H_2O_2 generated from 10^{-4} M PHA (i.e., 10^{-4} M H_2O_2) and the value of $k_{H_2O_2}$, the amount of $PhNO_2$ which could be generated from PhNO and H_2O_2 would account for $<10^{-3}$% of the observed $PhNO_2$ yield. Similarly the anaerobic oxidation of PHA with H_2O_2 is too slow to represent the source of $PhNO_2$; employing a 1 M solution of H_2O_2, several days were required for the reaction to proceed to completion. Addition of 1 M H_2O_2 did not effect k_{obs} or product distribution for O_2-dependent PHA oxidation, eliminating the possibility that H_2O_2 catalyzes the breakdown of an intermediate formed from PHA and O_2. Again, the absence of a lag phase during $PhNO_2$ formation suggests that H_2O_2 (formed from PHA oxidation) is not involved in the reaction sequence. Finally, catalase was shown not to significantly inhibit $PhNO_2$ formation. These observations rule out simple oxidizing species (e.g., O_2, H_2O_2) which may be considered responsible for $PhNO_2$ formation and prompt the hypothesis of a reactive

intermediate. Such an intermediate may have important mechanistic implications in describing amine carcinogenicity.

In summary, PHA oxidation proceeds with formation of PhNO, AzB, and $PhNO_2$, the latter not having been previously reported to be a reaction product. Evidence is presented suggesting that $PhNO_2$ formation proceeds through a reactive intermediate. Hydrogen peroxide has been ruled out as the reactive species. Finally, phosphate buffer has been shown to serve as a general catalyst for the oxidation sequence.

EXPERIMENTAL

Materials

PhNO, AzB, and $PhNO_2$ were obtained from Aldrich and purified prior to use; PhNO was recrystallized from $EtOH/H_2O$, AzB was recrystallized from CH_2Cl_2/pentane, and $PhNO_2$ was distilled (bp 78°C, 1.3 mm). PHA was prepared by the method of Smissman and Corbett (*19*) and freshly recrystallized from CH_2Cl_2/pentane prior to use. Metal-free buffers were prepared by dithiazone extraction (*20*) to avoid enhanced decomposition of PHA (*21*).

Methods

Reaction was carried out in an aspirator bottle (250 ml). A gas dispersion tube was introduced through the neck and the vacuum take-off was sealed with a rubber septum. Metal-free buffer (50 ml) was introduced into the reaction vessel, flushed with argon for 20 min and submerged in a bath thermostatted at 25°C. PHA (1–2 mg) was added and allowed to dissolve. The reaction mixture was then flushed with oxygen for 2 min with stirring. The gas dispersion tube was removed and the neck sealed with a gas inlet tube (connected to an oxygen line) positioned above the level of the reaction mixture. Oxygen pressure was thus maintained constant in the head space during the time reaction was monitored. Samples were taken at timed intervals through a rubber septum and analyzed by hplc. Components were separated on a Waters Bondapak C-18 column (30 cm × 4.6 mm, i.d.) using a mobile phase of 20 : 80, 2-propanol : water for PHA, PhNO, $PhNO_2$; azoxybenzene was eluted using a mobile phase of 65 : 35, methanol : water. A flow rate of 2 ml min^{-1} was maintained and effluent followed spectrophotometrically at 280 nm. Retention volumes for PHA, PhNO, and $PhNO_2$ were 5, 22.5, and 16.6 ml, respectively; retention volume for AzB with the stronger eluting solvent was 12 ml.

The kinetic studies were performed at pH's 6.75, 7.04, 7.25, and 7.40 at a minimum of three phosphate concentrations (0.01, 0.025 [pH 7.25 and 7.40], 0.05, and 0.10 *M* total phosphate) at 25°C (μ = 0.5, $NaClO_4$). The values of k_{obs} were obtained from plots of ln [(peak area)$_t$/(peak area$_\infty$] vs time. The oxygen concentration in all buffers was determined polarographically at 25°C with a YSI 5331 oxygen monitor equipped with a modified Clarke-type oxygen sensor

(Yellow Springs Instrument Co., Yellow Springs, Ohio) and found to be identical (2.2 m*M*) at all buffer concentrations and pH's used in this study.

The percentage of O_2 in artificial gas mixtures was determined by mixing known volumes of O_2 with known volumes of N_2, the volumes determined with a soap-film flow meter. The percentages of O_2 used were 0, 23, 42, 72, and 100.

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