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Chemistry

Oxidation of phenylhydroxylamine in aqueous solution: A model for study of the carcinogenic effect of primary aromatic amines

(arylhydroxylamine/carcinogenesis/kinetics)

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ABSTRACT **Phenylhydroxylamine is degraded in aqueous phosphate buffers at physiological pH values (6.8–7.4) to give nitrosobenzene, nitrobenzene, and azoxybenzene. The reaction is O_2 dependent and subject to general acid and general base catalysis. At pH ≤ 5.8 in cacodylate buffer, it is converted to *p*-nitrosophenol in addition to nitrosobenzene, nitrobenzene, and azoxybenzene. Nitrobenzene and *p*-nitrosophenol appear to form directly from phenylhydroxylamine. A common intermediate generated from phenylhydroxylamine and O_2 is suggested to account for the formation of nitrobenzene, nitrosobenzene, and *p*-nitrosophenol and is consistent with kinetic studies and ^{18}O-labeling experiments. The results suggest that neither hydrogen peroxide nor superoxide (O_2^-) are involved in the oxidation sequence.**

Arylhydroxylamines (AHs) are formed metabolically by the action of hepatic mixed-function oxidases on primary arylamines (1, 2). These compounds are of considerable interest because, on further reaction, they produce yet-undefined proximal carcinogens. Evidence supporting the involvement of ionic electrophilic species (1–4) and, alternatively, of free radical species (5–7) have been offered to describe the proximal carcinogen.

In preliminary *in vitro* metabolic studies in mammalian liver preparations with various AHs, their high reactivity has been confirmed and shown in large part not to depend on the presence of active enzyme or any proteinaceous material (8, 9). The chemistry of the AHs in several nonaqueous systems (10–12) and at pH extremes (13, 14) has been described; however, these transformations are highly medium dependent and cannot be extrapolated to aqueous systems and in addition, these studies were carried out at high concentrations of AH ($\geq 10^{-2}$ M) (11), at which the O_2 concentration is limiting.

Therefore, a more intensive investigation of the reactions of AHs in aqueous environments, particularly in the physiologically relevant pH range is warranted. In this report, we describe the chemistry of PHA in aqueous metal-free cacodylate (pH 5.3–6.8) and phosphate (pH 6.3–7.4) buffers. Although it is not a potent carcinogen, PHA was used because, structurally, it is the simplest representative of this class of compounds.

MATERIALS AND METHODS

Nitrosobenzene (PhNO), *p*-aminophenol (PAP), *p*-nitrosophenol (PNP), azoxybenzene (AzB), and nitrobenzene ($PhNO_2$) were from Aldrich and purified before use. PHA was synthesized by reduction of $PhNO_2$ with Zn metal (15) and recrystallized from CH_2Cl_2/C_5H_{12}. Because of the reported lability of hydroxylamines in the presence of heavy metals (11), the buffers were rendered metal free by extraction with dithizone solution (16). $^{18}O_2$ (99 atom %) and $H_2{}^{18}O$ (95 atom %) were from Prochem Isotopes (Summit, NJ).

Reactions were carried out in a 250-ml aspirator bottle fitted with a gas dispersion tube to introduce gas mixtures. The vacuum take off was sealed with a rubber septum and served as a sampling port.

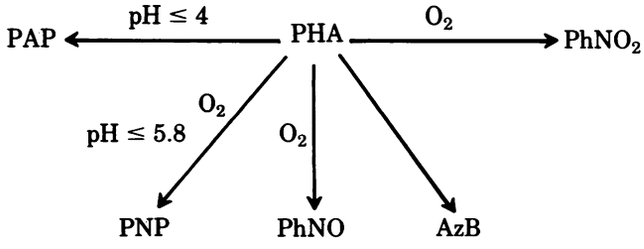
Metal-free buffer (50 ml) was introduced into the reaction vessel, which was flushed with argon for 20 min and submerged in a 25°C bath. PHA (1–2 mg) was added and allowed to dissolve, and the reaction mixture was then stirred and flushed with O_2 for 2 min. The gas dispersion tube was then removed and the neck of the flask was sealed with a rubber stopper containing a gas inlet tube (connected to an O_2 line) positioned above the level of the mixture. Oxygen pressure was thus maintained constant in the head space above the solution. Samples were taken at timed intervals by using a pressure-lock syringe through the rubber septum.

Components were separated on a Waters μBondapak RP-18 column (30 cm × 4.6 mm, i.d.) using a 2-PrOH/H_2O (20:80) mobile phase to elute PNP, PHA, PhNO, and $PhNO_2$ and a MeOH/H_2O (65:35) mobile phase to elute AzB. A flow rate of 2 ml/min was maintained, and the column effluent was monitored spectrophotometrically at 280 nm. Retention volumes for PHA, $PhNO_2$, PhNO, and PNP were 5.0, 16.6, 22.5, and 8.4 ml, respectively; AzB eluted in 12 ml of the stronger mobile phase.

Kinetic studies were carried out at 25°C at a minimum of five buffer concentrations at each pH studied, with ionic strength maintained constant at 0.5 with $NaClO_4$. k_{obs} values were determined from the slopes of plots of $\ln[(\text{peak area})_t/(\text{peak area})_\infty]$ vs. time (t).

RESULTS AND DISCUSSION

Previous studies have shown that PHA is unstable in solution, being oxidized to PhNO, which then condenses to AzB (10, 11, 17–19). Further investigation has now shown that, in oxygenated aqueous solutions (pH 6.0–7.8), PHA is also oxidized to $PhNO_2$. These products form only in the presence of O_2; in deoxygenated buffer (pH 5.3–7.8), PHA is stable. At pH ≤ 5.8, a fourth product, PNP is also formed. At pH ≤ 4.0, PHA also undergoes O_2-independent rearrangement to produce PAP. All products were characterized by their thin layer and high pres-



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Abbreviations: AH, arylhydroxylamine; PHA, phenylhydroxylamine; PhNO, nitrosobenzene; $PhNO_2$, nitrobenzene; AzB, azoxybenzene; PNP, *p*-nitrosophenol; PAP, *p*-aminophenol; HPLC, high-pressure liquid chromatography; SOD, superoxide dismutase.

sure liquid chromatographic behavior; mass spectra, which were identical to those of authentic samples; and elemental analyses, which were within acceptable limits (±0.4%).

Kinetic Studies. The loss of PHA (0.1 mM) from O_2-saturated aqueous buffer (pH 5.3–7.4; $[O_2]$ = 2.2 mM, determined polarographically) followed first-order kinetic behavior for a minimum of five half-lives. The dependence of PHA disappearance on O_2 concentration was determined by measuring PHA concentration as a function of time at several predetermined O_2 concentrations. A plot of log$k$ vs log$[O_2]$ was linear with slope of ≈0.93.

A high pressure liquid chromatography (HPLC) method was developed that permits simultaneous monitoring of substrate disappearance and specific appearance of each degradation product. The distribution of products present in an incubation mixture of PHA in cacodylate buffer is shown in Fig. 1 (similar behavior was observed in phosphate buffer). PhNO forms initially by direct action of O_2 on PHA (11) and is then consumed by conversion to AzB.

$PhNO_2$ formation exhibited no lag phase and followed first-order kinetics; the observed rate constant for its appearance was 92–98% of that for PHA disappearance, suggesting that $PhNO_2$ forms directly from PHA (scheme I) rather than from PhNO.

At pH ≤ 5.8, PNP was also produced. Its appearance followed apparent first-order kinetics and had an observed rate constant of 80–100% of that for PHA loss. Based on these observations, it appears that PNP also forms directly from PHA.

Buffer Catalysis. The rate of PHA degradation was dependent on buffer concentration in both cacodylate and phosphate buffers. Plots of $k_{obs}/[O_2]$ vs. total buffer concentration (not shown) were linear, with nonzero intercepts, suggesting that PHA disappearance proceeds through both buffer-catalyzed (k_B) and buffer independent (k_{OX}) pathways, as described by Eq. 1,

$$k_{obs} = (k_B B_T + k_{OX})[O_2], \quad [1]$$

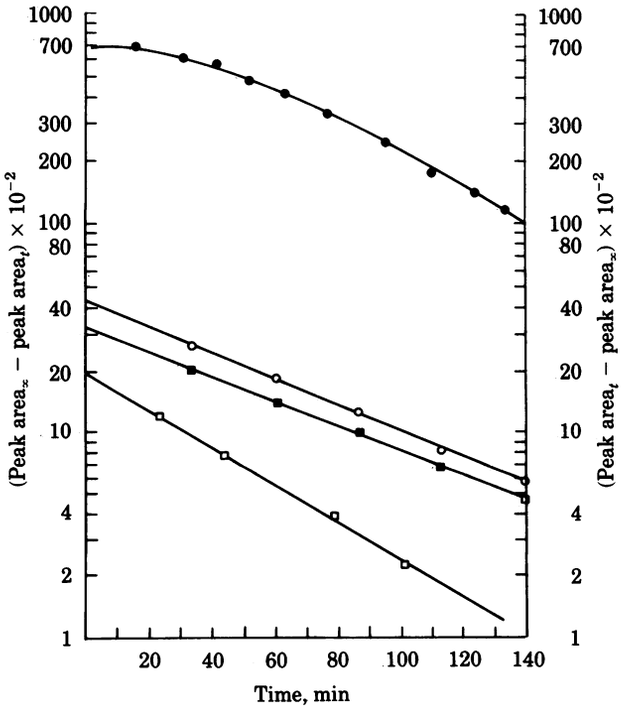


FIG. 1. Distribution profile. PHA (○) disappearance (calculated as peak area$_t$ − peak area$_\infty$, where peak area$_\infty$ = 0) and AzB (●), PNP (■) and $PhNO_2$ (□) appearance (calculated as peak area$_\infty$ − peak area$_t$) 0.01 M cacodylate buffer (pH 5.3; μ = 0.5, $NaClO_4$) at 25°C.

Table 1. Kinetic parameters for PHA oxidation

Buffer	$k_0 \times 10^1$, M^{-1} sec^{-1}	$k_{HO} \times 10^{-5}$, M^{-2} sec^{-1}	k_{ga} M^{-2} sec^{-1}	k_{gb} M^{-2} sec^{-1}
Phosphate	1.5	2.3	2.3	2.3×10^5
Cacodylate	0.6	2.3	3.5	4.1

PHA was incubated in the appropriate O_2-saturated buffer (five buffer concentrations at each pH), and substrate disappearance was monitored by HPLC. k_0 and k_{HO} were determined graphically from Eqs. 1 and 2. k_{ga} and k_{gb} were determined graphically from Eq. 5 for cacodylate buffers and from Eqs. 7 and 8 for phosphate buffers. Values represent the average of triplicate determinations.

where B_T is total buffer concentration. Plots of k_{OX} vs. $1/a_H$ were linear but had nonzero intercepts, suggesting that k_{OX} is a composite of a hydroxide-dependent (k_{HO}) and a pH-independent (k_0) term as described by Eq. 2,

$$k_{OX} = k_{HO} K_w a_H^{-1} + k_0, \quad [2]$$

where a_H is the activity of H_3O^+, as measured with a glass electrode. k_{HO} was calculated from the slope of plots of k_{OX} vs. a_H^{-1} and was identical for cacodylate and phosphate buffers, lending credibility to comparisons of these two systems (Table 1). k_0 was determined from the intercepts of these plots and was 0.15 M^{-1} sec^{-1} and 5.9 × 10^{-2} M^{-1} sec^{-1} in phosphate and cacodylate buffers, respectively. The inconsistency in k_0 values between the two buffer systems (a difference of a factor of 2.5) probably reflects the inaccuracy inherent in calculation or a minor kinetic salt effect [a factor of 2.5 in k_0 corresponds to an energy difference of <0.55 kcal mol^{-1} (1 cal = 4.18 J)].

The buffer-dependent term ($k_B B_T$; see Eq. 1) was, in both buffer systems, assumed to consist of a general acid (k_{ga}) and a general base (k_{gb})-catalyzed component, as described by Eq. 3,

$$k_B = k_{ga}[HX] + k_{gb}[X^-], \quad [3]$$

where HX and X^- are the acid and conjugate-base forms of the buffer, respectively.

Catalysis by Cacodylate Buffer. From Eqs. 1 and 3, an expression (for k_{obs}) for PHA degradation in *cacodylate buffer* is generated (Eq. 4),

$$k_{obs}/[O_2] = k_{OX} + (k_{ga}[HA] + k_{gb}[A^-]), \quad [4]$$

where HA and A^- are cacodylic acid and cacodylate, respectively. Rearranging Eq. 4 and expressing HA and A^- in terms of total cacodylate (C_T) concentration gives Eq. 5,

$$k_{obs} = \left[\left(k_{ga}\frac{a_H}{K_a + a_H} + k_{gb}\frac{K_a}{K_a + a_H}\right) C_T + k_{OX}\right][O_2], \quad [5]$$

where K_a is the dissociation constant for cacodylic acid [$10^{-6.2}$ (20)].

k_B is given by the slope (slope A) of plots of $k_{obs}/[O_2]$ vs. C_T, and, from it, k_{gb} and k_{ga} can be determined graphically (Fig. 2). This treatment of the kinetic data supports the hypothesis that buffer catalysis by cacodylate involves both general-acid and general-base terms (or kinetically equivalent expressions).

Catalysis by Phosphate Buffer. A similar approach was taken to describe buffer catalysis by *phosphate*. However, this system is complicated by the polyprotic nature of the buffer species. A kinetic expression for k_{obs} consistent with the experimental data is given by Eq. 6,

$$k_{obs} = (k_1[H_2PO_4^-] + k_2[PO_4^{3-}] + k_{OX})[O_2], \quad [6]$$

where k_1 and k_2 are the general acid and general base rate constants, respectively. Kinetically equivalent terms for $H_2PO_4^-$

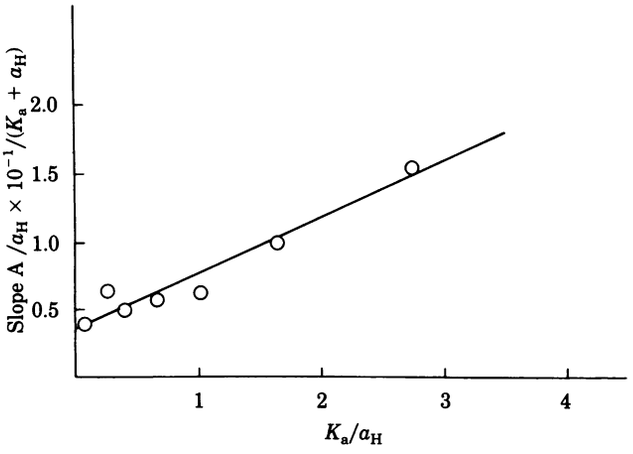


FIG. 2. Secondary plot of slope $A/a_H(K_a + a_H)^{-1}$ vs. (K_a/a_H) for oxidation of PHA in cacodylate buffer. The theoretical line was computer generated by a least-squares program.

and PO_4^{3-} can be written that, at this time, cannot be eliminated from consideration. The validity of this equation was established graphically by rearranging it and expressing $H_2PO_4^-$ and PO_4^{3-} in terms of total phosphate concentration $[P_T]$ to give Eq. 7,

$$k_{obs}/[O_2] = \left(\frac{k_1 a_H^2 + k_2 K_{a_2} K_{a_3}}{a_H^2 + K_{a_2} a_H + K_{a_2} K_{a_3}}\right) P_T + k_{OX} \qquad [7]$$

where K_{a_2} is the acid dissociation constant for $H_2PO_4^-$ ($10^{-6.5}$; 20) and K_{a_3} is the acid dissociation constant for HPO_4^{2-} ($10^{-12.3}$; 21). From plots of $k_{obs}/[O_2]$ vs. P_T, the buffer-independent and buffer-dependent terms were determined. Over the pH range studied (6.75–7.40), $a_H^2 + K_{a_2} a_H \gg K_{a_2} K_{a_3}$, which simplifies the expression for the slope (slope B) to one (Eq. 8) that can be solved

$$\frac{\text{slope B}}{a_H/(K_{a_2} + a_H)} = \frac{k_2 K_{a_2} K_{a_3}}{a_H^2} + k_1 \qquad [8]$$

graphically. The intercept of this secondary plot (Fig. 3), k_1, is the third-order rate constant for general acid catalysis by $H_2PO_4^-$, and the slope gives k_2, the rate constant for general base catalysis by PO_4^{3-} (see Table 1). Similarly, analyzing the kinetic data by using a mathematical SIMPLEX scheme in which all possible phosphate species were considered gave an equation independent of $[HPO_4^{2-}]$, further supporting the hypothesis that a term in $[HPO_4^{2-}]$ (or a kinetically equivalent expression) does not contribute to the overall rate expression. General acid catalysis by $H_2PO_4^-$ (and the absence of a term in HPO_4^{2-}) is also supported by the data for cacodylic acid, a monoprotic acid that has a dissociation constant ($10^{-6.2}$) similar to that for $H_2PO_4^-$ ($K_a = 10^{-6.5}$). The rate constants for general acid catalysis (k_{ga}) of PHA disappearance by phosphate and cacodylate buffers were of similar magnitudes and, therefore, in accord with the Brønsted relationship, support the hypothesis that $H_2PO_4^-$ or kinetically equivalent species (rather than HPO_4^{2-}) is the general acid catalyst.

Similarly, data from studies with cacodylate buffers suggest the definitive absence of general base catalysis by HPO_4^{2-} or kinetically equivalent species. Cacodylate behaves as a classical general base (20) and, because HPO_4^{2-} and cacodylate are oxygen bases of similar strength (their pk'a's differ by 0.3 log units), they would be expected to have similar general-base-catalysis activities. Nevertheless, no measurable $k_{gb}[HPO_4^{2-}]$ term could be observed. In fact, the kinetics require the involvement of a much stronger general base catalyst—i.e., phosphate trianion ($K_a = 10^{-12.3}$) or a kinetically equivalent species (despite its low concentration relative to $[HPO_4^{2-}]$ at the pH values studied). Using a Brønsted relationship to compare the catalytic effectiveness of PO_4^{3-} vs. cacodylate (see Table 1) suggests that general base catalysis of PHA degradation should be highly dependent on the basicity of the catalyst ($\beta \approx 0.8$) if PO_4^{3-} is the effective species. This value of β is mechanistically reasonable.

The formation of both $PhNO_2$ and PNP was subject to buffer catalysis in addition to proceeding via buffer-independent pathways [i.e., plots of k_{obs} for $PhNO_2$ or PNP formation vs. total buffer concentration (phosphate and cacodylate for $PhNO_2$ and cacodylate at pH 5.3 for PNP) were linear and had nonzero intercepts] (Table 2). The buffer-dependent ($k_B = 1.6\ M^{-2}\ sec^{-1}$) and buffer-independent ($k_{OX} = 9.7 \times 10^{-2}\ M^{-1}\ sec^{-1}$) rate constants for PNP formation at pH 5.3 in cacodylate buffer were similar in magnitude to the corresponding ones for PHA dis-

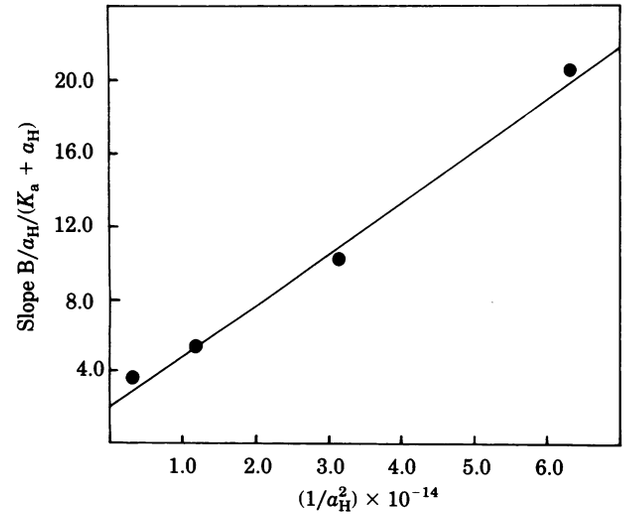


FIG. 3. Secondary plot of slope $B/a_H(K_a + a_H)^{-1}$ vs. a_H^{-2} for the oxidation of PHA in phosphate buffer. The theoretical line was computer generated by a least-squares program.

Table 2. Kinetic parameters for nitrobenzene formation

	$PhNO_2$ appearance		PHA disappearance	
pH	$k_{OX} \times 10^2$, $M^{-1}\ sec^{-1}$	k_B^*, $M^{-2}\ sec^{-1}$	$k_{OX} \times 10^2$, $M^{-1}\ sec^{-1}$	k_B^*, $M^{-2}\ sec^{-1}$
	Cacodylate buffer			
5.3	4.0	3.8	4.2	3.7
5.8	5.7	3.4	8.0	3.4
6.0	6.6	3.1	4.6	3.2
6.6	8.0	3.0	7.3	3.5
6.8	10.2	2.9	9.6	3.7
	Phosphate buffer			
6.8	11.3	2.3	11.9	2.5
7.0	12.8	3.0	13.5	3.1
7.2	13.8	2.8	13.9	3.2
7.4	10.6	29.2	12.8	35.2

PHA was incubated in the appropriate O_2-saturated buffer (five buffer concentrations at each pH), and substrate disappearance and $PhNO_2$ appearance were monitored by HPLC. Buffer-dependent (k_B) and independent (k_{OX}) rate constants were determined graphically from the equation $k_{obs} = (k_{OX} + k_B B_T)[O_2]$. Values represent the average of triplicate determination.

* Composite of k_{ga} and k_{gb}.

appearance. The data were insufficient to further define the buffer catalytic terms(s).

The observed rate constants for PHA disappearance (k_{obs}) were approximately equal to those for $PhNO_2$ formation (see Table 2) in both buffers. Application of Eq. 5 to the data in Table 2 suggests that formation of $PhNO_2$ is subject to general acid and general base catalysis. The occurrence of general catalysis of $PhNO_2$ formation and the similarity in the rate constants for PHA disappearance and $PhNO_2$ formation further substantiate the hypothesis of direct formation of $PhNO_2$ from PHA.

Product Distribution. PHA was incubated in phosphate and in cacodylate buffers (at all pHs studied in the kinetic experiments and constant buffer concentration) for seven half-lives, and the final product yields were determined by HPLC. The product distribution (Table 3) showed no simple correlation with the degree to which the kinetic terms of Eqs. 4 and 6 contribute to the rate of PHA degradation, which suggests that rates and products are determined in different processes and that, therefore, there must be at least one intermediate in the reaction sequence. In phosphate buffer, catalysis was not associated with the condensation reaction; as the extent of buffer catalysis increased, the AzB yield decreased. Phosphate must catalyze the oxidation reaction by participating in the initial step or in the breakdown of an intermediate involved in the rate-determining step.

A much higher percentage of PHA is converted to PhNO in cacodylate buffer than in phosphate buffer (see Table 3). Also, the nominal loss in participation of kinetic pathways involving buffer terms as the pH increases from 5.2 to 6.8 cannot readily account for the large decreases in $PhNO_2$ and AzB yields observed. Clearly, the overall mechanism is very complicated and cannot yet be defined.

Mechanisms for $PhNO_2$ and PNP Formation. Several observations suggest that $PhNO_2$ and PNP form independently of AzB and PhNO: (*i*) both PhNO and AzB are stable in O_2-saturated phosphate and cacodylate buffers, (*ii*) no simple relationship exists between the kinetic terms of Eqs. 4 and 6 and the measured yields of $PhNO_2$ and PNP, and (*iii*) the production of $PhNO_2$ and PNP proceeds with apparent first-order kinetic behavior. A lag phase would be anticipated if PhNO, AzB, or some other compound was a precursor to PNP or $PhNO_2$ formed in the rate-determining step. Although this lag phase would not be observed if the final oxidation step was fast relative to the rate-determining step, under these conditions, a proportionality would exist between the concentration of PNP or $PhNO_2$ and that of PhNO or AzB. Neither of these situations was observed, however; thus, it appears that PNP and $PhNO_2$ form directly from PHA. Their production is, however, dependent on the presence of O_2 (i.e., in an O_2-free atmosphere, PHA is stable).

Bamberger (17) has reported that the autoxidation of PHA generates hydrogen peroxide, and the involvement of H_2O_2 as an oxidizing agent in producing $PhNO_2$ or PNP has been investigated. PhNO is quantitatively converted to $PhNO_2$ by H_2O_2, and the rate of disappearance of PhNO is equal to the rate of $PhNO_2$ production. However, the rate constant for this reaction ($k_{H_2O_2} = 2.8 \times 10^{-5}\ M^{-1}\ sec^{-1}$) is too small to account for the observed yield of $PhNO_2$. The possibility that $PhNO_2$ is produced by H_2O_2 oxidation of PHA was similarly investigated; but ruled out based on several observations. (*i*) Although H_2O_2 quantitatively converts PHA to $PhNO_2$ (in the absence of O_2), this reaction takes several days to reach completion and is, therefore, too slow to represent the pathway for $PhNO_2$ formation. (*ii*) In an O_2-containing atmosphere, addition of 1 M H_2O_2 to phosphate buffer (pH 6.8) containing PHA failed to change k_{obs} for PHA disappearance or for $PhNO_2$ formation nor did it effect product distribution. (*iii*) $PhNO_2$ production follows first-order kinetic behavior with no lag phase, as would be anticipated if H_2O_2 was involved in $PhNO_2$ generation. (*iv*) Addition of catalase did not inhibit PHA loss nor $PhNO_2$ formation. Thus, the involvement of H_2O_2 in $PhNO_2$ or PNP formation seems highly unlikely.

Superoxide anion (O_2^-) was investigated as an alternative oxidizing agent for the production of $PhNO_2$ and PNP. It reacted instantaneously in buffer (pH 6.4–7.4) with PHA or PhNO to form $PhNO_2$ in quantitative yield. To clarify its role, the effect of superoxide dismutase (SOD), an enzyme that catalyzes disproportionation of O_2^- to O_2 and H_2O_2 (22, 23), on the kinetics of PHA disappearance and $PhNO_2$ formation was studied. SOD (1 mg/25 ml of buffer) failed to retard $PhNO_2$ formation in PHA incubations at pH 7.2 or pH 7.4 and in fact, had a slight accelerating effect (a factor of 3) on the rate of PHA disappearance. However, a similar quantity of SOD that had been denatured by heating at 100°C for 5 min failed to produce a similar acceleration of degradation, which suggests that the metal ions associated with the enzyme are not responsible for the acceler-

Table 3. Contributions of kinetic pathways to degradation of PHA (defined as K_{obs}) and relative yields of products

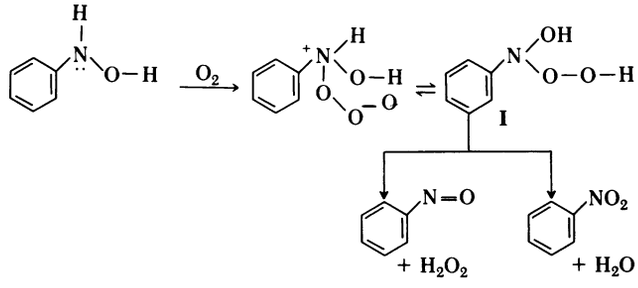
	Phosphate buffer, pH					Cacodylate buffer, pH			
	6.31	6.83	7.24	7.40	7.84	5.30	6.00	6.40	6.84
	% of degradation proceeding through pathway								
Buffer catalyzed	48	43	49	55	69	85.4	85.3	85.0	83.5
General acid catalyzed	46	27	10	7	1	78.4	59.0	39.7	20.5
General base catalyzed	2	16	39	48	68	7.0	26.3	45.3	63.0
Buffer independent	52	57	50	44	31	14.6	14.6	14.9	16.5
Specific base catalyzed	2	5	10	12	16	0.1	0.5	1.3	3.5
pH independent	50	52	40	32	15	14.5	14.1	13.6	13.0
	Product yield after seven half-lives, %								
$PhNO_2$	12	12	12	11.5	10.5	8.8	3.7	3.6	2.2
A_2B	20	16	10.5	8	5	5.4	2.4	2.0	1.3
PhNO*	68(46)	72(54)	77.5(56)	80(60)	84.5(69)	70.1(60.1)	93.7(78.4)	94.5(79.5)	96(84.4)
PNP	—	—	—	—	—	15.6	—	—	—

Weighed amounts of PHA were incubated in buffer (five buffer concentrations at each pH) at 25°C for seven half lives. The disappearance of substrate and appearance of products were determined by HPLC, and rate constants [according to $k_{obs} = (k_1[HA] + k_2[A^-] + k_{OX})[O_2]$ and $k_{OX} = k_{HO}k_w a_H^{-1} + k_0$)] and product yields were determined. All values represent the average of triplicate determinations.

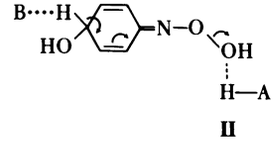
* Numbers in parentheses are measured, *uncorrected* yields of PhNO. Due to the observed mechanical loss of PhNO from the system, its yield was corrected to bring total product yield to 100%. Control experiments indicated a physical loss of ≈20% PhNO during the course of a typical kinetic run.

ating effect observed with the active enzyme. The rate enhancement depended linearly on the concentration of active enzyme used and occurred only in an O_2-containing atmosphere (i.e., PHA was stable in an argon atmosphere in the presence of SOD). Although the effect of SOD on PHA degradation cannot be adequately explained at this time, the lack of an inhibitory effect suggests that superoxide does not participate in the reaction sequence.

By elimination of these simple oxidizing species, it appears that a reactive intermediate, perhaps one forming between O_2 and PHA, is responsible for generating $PhNO_2$ and PNP. The direct involvement of O_2 in the formation of $PhNO_2$ was determined by carrying out the oxidation of PHA in phosphate buffer (0.1 M, pH 7.2, $\mu = 0.5$) in an environment supplemented with ^{18}O-labeled O_2. The $PhNO_2$ formed in this experiment was enriched to an extent of 82 ± 4% with one atom of ^{18}O per molecule, as determined by isotope-ratio mass spectrometry (M+, 123 and 125). Doubly-labeled $PhNO_2$ (M+ 127) was not formed. A similar experiment with $^{16}O_2$ in buffer enriched with $H_2{}^{18}O$ resulted in formation of $PhNO_2$ with no ^{18}O incorporation. Thus, $PhNO_2$ appears to form by direct involvement of O_2 with PHA to give of a reactive oxygenated intermediate that subsequently collapses to yield product. A possible mechanism for $PhNO_2$ formation is shown below, in which elimination of water from the oxygenated intermediate, **I**, produces $PhNO_2$. The PhNO formed in these reactions contained no ^{18}O



label, suggesting that its formation either does not involve an oxygenated intermediate (but rather dehydrogenation of PHA) or that the oxygenated intermediate eliminates the elements of H_2O_2 to form PhNO. **I** is also consistent with the O_2-dependent formation of PNP. Attack of water (or HO^-) at the para position of this intermediate concomitant with (or subsequent to) buffer/solvent-assisted expulsion of HO^- would give **II**, which, on elimination of the elements of water, gives PNP.



II

This study has shown that, once formed, arylhydroxylamines degrade spontaneously (under aerobic conditions) to form potentially carcinogenic products (24) through intermediates that are also potentially carcinogenic. These reactions are buffer catalysed and the occurrence of catalysis by phosphate (a physiological buffer) suggests that similar acceleration of AH oxidation may occur *in vivo*. The role of such nonenzymatic reactions and, in particular, the reactive intermediates formed in the course of AH oxidation (e.g., **I** and **II**) in precipitating oncogenic responses deserves further investigation.

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