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Nonenzymatic Reduction of Nitrosobenzene to Phenylhydroxylamine by NAD(P)H

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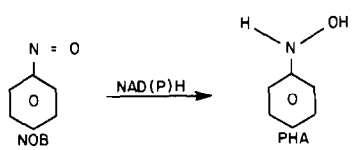
The nonenzymatic reduction of nitrosobenzene by NADPH and NADH in aqueous buffer solution at 25°C is described. Both reactants quantitatively convert nitrosobenzene to phenylhydroxylamine. Rate constants for reduction (k_r) were determined spectrophotometrically and found to be identical at pH 5.7 and 7.4 and independent of buffer concentration. The values of k_{NADH} (124–149 M^{-1} sec^{-1}) and k_{NADPH} (131–170 M^{-1} sec^{-1}) are essentially identical. The reaction is not subject to general catalysis or specific salt effects. The oxidation of phenylhydroxylamine by NAD(P) to nitrosobenzene is only stimulated by a factor of 1.2 over oxidation in its absence (when the ratio of NADP : phenylhydroxylamine was 8 : 1).

INTRODUCTION

The metabolic formation of arylhydroxylamines from arylamines appears to be a prerequisite for the expression of the carcinogenesis of the parent amine (*1–3*). *In vivo* the *N*-hydroxylamines are rapidly converted to their nitroso analogs by ferrihemoglobin (*4, 5*) and through oxygen-mediated nonenzymatic oxidation (*6, 7*). In the presence of a flavoprotein-dependent reductase and NADPH, an enzymatic pathway has been found for the reduction of 2-nitrosonaphthalene to 2-naphthylhydroxylamine (*7*), and of other nitroso derivatives (*8*).

Our current research efforts, directed toward study of the nonenzymatic oxidation of primary arylhydroxylamines, prompted an investigation of the nonenzymatic reduction of nitrosobenzene (NOB) in aqueous solution by NADH and NADPH. Conversion of nitroso derivatives to arylhydroxylamines may represent an important pathway for the production of proximal carcinogenic intermediates, in light of evidence presented by Miller *et al.* (*1, 2, 9*) showing the potential alkylating ability (toward nucleic acids) of hydroxylamines in the presence of esterifying enzymes (*10*). In this study NAD(P)H was shown to reduce quantitatively NOB to PHA (Eq. [1]).



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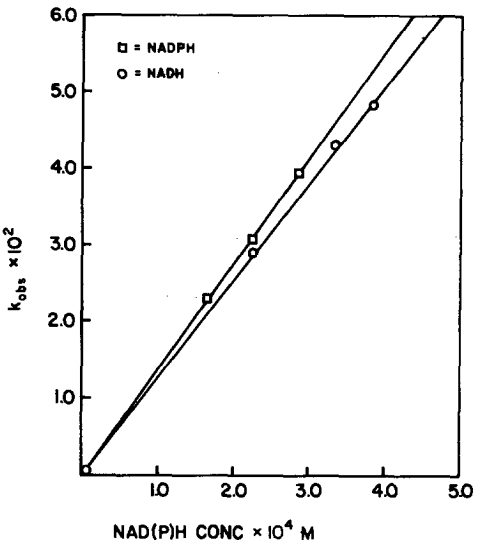

FIG. 1. k_{obs} vs [NAD(P)H] for the reduction of nitrosobenzene in 0.01 *M* acetate buffer (pH 5.7, 25°C, μ = 0.5, $NaClO_4$).

EXPERIMENTAL

NADH, NADPH, NAD, and NADP were obtained from Sigma Chemical Company (St. Louis, Mo.) and used without further purification. Nitrosobenzene (NOB) was obtained from Aldrich Chemical Company (Milwaukee, Wis.) and recrystallized from methylene chloride–pentane prior to use. Phenylhydroxylamine was prepared by reduction of nitrobenzene with zinc and ammonium chloride (*11*) and recrystallized from methylene chloride–pentane. Kinetic studies were performed on a Cary 219 spectrophotometer thermostated at 25°C. Stock solutions of NOB were prepared in 0.01 and 0.10 *M* metal-free (*12*) buffers (μ = 0.5, $NaClO_4$) which were degassed with nitrogen; 3.0 ml of this solution was pipetted into a cuvette containing NAD(P)H accurately weighed on a Cahn electrobalance. The disappearance of NOB was followed spectrophotometrically at 308 nm (vs an air blank). The initial concentration of NOB in the cuvette was ca. 1.7×10^{-5} *M* while the initial NAD(P)H concentration was varied from 1.8 to 4.0×10^{-4} *M*. To calculate rate constants, reactions were followed for four to five half-lives. The actual values of OD_t and OD_∞ for the first-order process were obtained by feathering (method of residuals) (*13*) because of a continuous zero-order decrease in absorbance due to decomposition of NAD(P)H both during NOB reduction and after t_∞ for the reduction reaction. The values of k_{obs} were obtained from plots of ln ($OD_t - OD_\infty$) vs time. Data were obtained at pH 5.7 and 7.4 in acetate (0.01 and 0.10 *M*) and phosphate (0.01 and 0.10 *M*) buffers, respectively, employing four concentrations of NAD(P)H at each buffer concentration.

Product studies were carried out by high-performance liquid chromatography

TABLE 1

SECOND-ORDER RATE CONSTANTS (M^{-1} SEC^{-1}) FOR THE REDUCTION OF NITROSOBENZENE BY NADH AND NADPH IN AQUEOUS BUFFER AT 25°C (μ = 0.5, $NaClO_4$)

pH	Buffer	k_{NADH}	k_{NADPH}
5.7	0.10 M_T HOAc–NaOAc	124	148
	0.01 M_T HOAc–NaOAc	126	139
7.4	0.10 M_T NaH_2PO_4–Na_2HPO_4	149	170
	0.01 M_T NaH_2PO_4–Na_2HPO_4	139	131

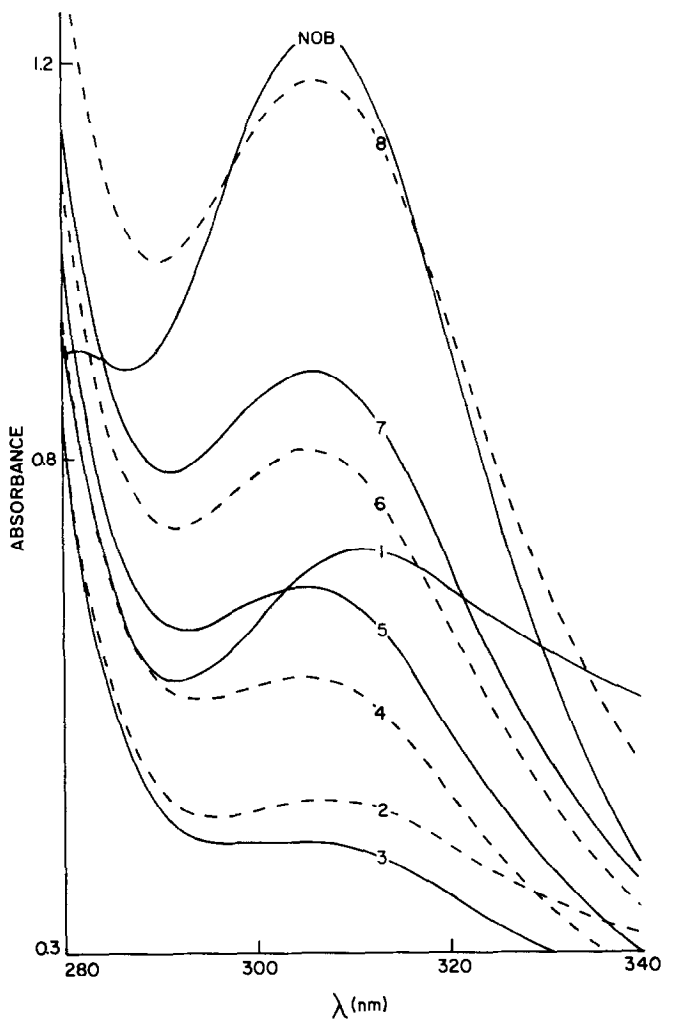

FIG. 2. Absorption spectra generated after mixing NOB (1.40×10^{-4} M) and NADPH (1.35×10^{-4} M in acetate buffer; 0.1 M; pH 5.7; μ = 0.5, $NaClO_4$) at 25°C. Spectra obtained (1) 0.6 min, (2) 3 min, (3) 6 min, (4) 39 min, (5) 56 min, (6) 88 min, (7) 120 min, (8) 11 hr after mixing. Spectrum labeled NOB is that of NOB in the buffer (in the absence of NADPH).

(hplc) and uv spectroscopy. The hplc analysis was carried out on a component system consisting of a Waters Model U6K injector, Model 6000A pump, and Model 440 uv detector (Waters Associates) employing an RP-18 column (Waters, μBondapak C_{18}). A mobile phase of 20% 2-propanol–80% water was used at a flow rate of 2.0 ml/min. Retention volume of PHA was 4.8 ml and for NOB was 19.0 ml. The concentration of PHA was monitored spectrophotometrically at 280 nm as a function of peak height on a strip chart recorder.

The uv spectroscopic product analysis was performed in the presence of oxygen by obtaining a uv spectrum of NOB (1.4×10^{-4} *M*) in pH 5.7 acetate buffer. NOB was found to be stable and exhibit a useful λ_{max} for study at 308 nm. NADPH (0.36 mg) was added to the cuvette containing NOB to yield a final concentration of NADPH equal to 1.35×10^{-4} *M*. The absorption spectrum of the solution was monitored at various time points (0.6, 3, 6, 40, 60, 90, 120 min, and 11 hr); several accompanying hplc analyses of the reaction components were carried out. When these uv scans indicated the reaction was complete, an additional amount of NADPH was added to the same cuvette and the uv scans were resumed until no further absorbance changes occurred.

The possible nonenzymatic oxidation of PHA by NADP in the same acetate buffer in the presence of oxygen was investigated kinetically by hplc (same mobile phase and conditions as for product studies). Approximately 0.20 mg of PHA was added to each of two volumetric flasks ("D" and "E"). NADP (0.77 mg) was added to D and both flasks were diluted with buffer to yield solutions which were 3.6×10^{-5} *M* in PHA; solution D was also 2.9×10^{-4} *M* in NADP. The relative rates of disappearance of PHA in the two solutions were monitored by hplc analysis at regular time intervals.

RESULTS

The reduction of NOB by either NADH or NADPH follows pseudo-first-order kinetics in accord with Eq. [2], as determined by the linear fit of the ln of the absorbance changes vs time over five half-lives. Under the experimental conditions,

$$-\frac{d[\text{NOB}]}{dt} = k_{obs}[\text{NOB}] \qquad [2]$$

where k_{obs} (sec^{-1}) can be expressed by Eq. [3] where k_r (M^{-1} sec^{-1}) is

$$k_{obs} = k_r[\text{NAD(P)H}], \qquad [3]$$

the second-order rate constant for reduction by either NADH or NADPH (*14*). As Eq. [3] suggests, plots of k_{obs} vs [NAD(P)H] were linear with zero intercept (Fig. 1). NOB was stable in the absence of NAD(P)H. The slopes of these plots yield the value of k_r at the pH and buffer concentration of the experiment. The values of k_{NADH} and k_{NADPH} for 0.01 and 0.10 *M* acetate and phosphate buffers are given in Table 1. Both rate constants were independent of pH (in the range studied: 5.7–7.4), and buffer concentration. In addition, the values of k_{NADH} and k_{NADPH} are

essentially identical. One series of kinetic runs in 0.10 *M* acetate substituting NaCl for $NaClO_4$ (for maintenance of ionic strength) yielded an identical value of k_{NADH} indicating the absence of a specific salt effect upon the reduction.

The chemical reduction of NOB by NAD(P)H produces only PHA as determined by quantitative hplc analysis employing authenic PHA as an external standard. No other uv-detectable products were observed. Additional support for the formation of PHA was obtained from uv spectrophotometric analysis carried out under aerobic conditions. A uv spectral scan of a solution of NOB after the addition of approximately an equimolar equivalent of NADPH (Fig 2) reveals a shift in λ_{max} from 308 nm (λ_{max} of NOB) to 312 nm (λ_{max} of PHA) with an accompanying decrease in absorbance. Additional uv scans 3 and 6 min after addition of NADPH showed a continual drop in absorbance. After ca. 40 min the absorbance began to increase with the λ_{max} shifting back to 308 nm (Fig. 2). The hplc analysis of this solution showed the presence of PHA, NOB, and nitrobenzene (*15*). With subsequent scans the absorbance at 308 nm continued to increase. The hplc analysis at ca. 60 min after initiation showed further increases in the concentration of NOB and nitrobenzene with a decrease in the concentration of PHA. The presence of nitrobenzene as a product of PHA oxidation was confirmed based on its retention behavior on an RP-18 or a μBondapak NH_2 hplc column using a range of methanol/water and 2-propanol/water ratios as mobile phases. In all cases behavior was identical to that of an authentic sample of nitrobenzene. In addition, an electron-impact mass spectrum of the compound corresponding to the hplc peak assumed to arise from nitrobenzene was identical with the mass spectrum of an authentic sample of nitrobenzene. The hplc analysis ca. 11 hr after initiation of reaction showed only NOB and nitrobenzene remaining (*16*). An additional amount of NADPH was then added to the sample cuvette; the same spectral pattern was again observed, but with smaller absorbance changes due to

TABLE 2

YIELD OF PHENYLHYDROXYLAMINE (PHA) GENERATED FROM THE NADH REDUCTION OF NITROSOBENZENE (NOB) IN 0.10 *M* ACETATE BUFFER (pH 5.7, 25°C, μ = 0.5 $NaClO_4$)

Solution[a]	[NOB][b] (× 10^5 *M*)	NADH[c] (mg/10 ml)	[PHA][d] (× 10^6 *M*)	Percentage yield
A_1	1.03	0.90	9.16	82
A_2	1.03	0.45	8.42	89
B_1	1.16	0.90	9.75	84
B_2	1.16	0.45	9.90	85
C_1	1.38	0.90	13.2	96
C_2	1.38	0.45	12.8	93

[a] Solutions were prepared in 0.1 *M* acetate buffer (pH 5.7, μ = 0.5, $NaClO_4$) and degassed with nitrogen.

[b] Initial concentration of nitrosobenzene employed in the reduction.

[c] Initial amount of NADH in reaction mixture.

[d] Determined by hplc analysis; measuring peak height of PHA band and comparing with an external standard. Represents the average of three determinations.

the now smaller initial concentration of NOB. Under these conditions, nitrobenzene was not reduced by NAD(P)H. Spectral changes arising from NAD(P)H/NAD(P) absorption did not interfere with monitoring at the selected analytical wavelengths. A detailed hplc product study under anaerobic conditions indicated that PHA was formed in 85–95% yield (see Table 2) by NAD(P)H reduction of NOB. The only other uv-detectable compound present in the mixture was NOB.

First-order plots of the oxidation of PHA in the presence and absence of NADP were linear (reactions were followed to more than two half-lives). The absolute values of the rate constants obtained from the slopes of such plots ($k_{obs,N} = 6.8 \times 10^{-5}$ sec^{-1} in the presence of NADP; $k_{obs,0} = 5.8 \times 10^{-5}$ sec^{-1} in the absence of NADP) are not meaningful, since constant oxygen concentration was not maintained; but the ratio of the $k_{obs}(k_{obs,N}/k_{obs,0})$ values are significant, since both runs were performed under identical experimental conditions. The results indicate that the oxidation of PHA occurred at a 1.2-fold greater rate when NADP was present ([NADP]:[NOB] = 8.1) than in its absence, but this difference may not be biologically important.

DISCUSSION

Many studies of the enzymatic reduction of aromatic nitroso compounds to the corresponding *N*-hydroxylamine employing NADPH as a cofactor have been reported (*7, 8*). Indeed, many of the studies have employed nitrosobenzene (*17–21*), the compound under study in this communication. The enzyme which catalyzes this reduction (diaphorase) requires NADPH as cofactor (*17, 21*). To our knowledge, this is the first conclusive demonstration that the reduction of NOB to PHA by either NADPH or NADH can occur nonenzymatically. Berheim (*22*) observed the nonenzymatic oxidation of NADPH by nitrosobenzene but did not determine kinetic constants nor clearly elucidate the nature of the reaction products. Because she was monitoring NADPH oxidation rather than NOB, biphasic kinetics were observed. This result can now be explained as a rapid initial decrease in NADPH concentration due to its oxidation by NOB and then an apparent slower rate of NADPH loss due to the replenishing of NOB by the air oxidation of PHA. A similar explanation was postulated by Bernheim but could not be verified using the analytical methodology employed in the earlier study. The values of the rate constants for reduction (Table 1) indicate that NADH and NADPH are equally effective as reducing agents for NOB. A 10-fold increase in buffer concentration employing acetate (pH 5.7) or phosphate (pH 7.4) buffer shows no effect on the value of the rate constant, suggesting that the reduction by either NADPH or NADH is not subject to general catalysis. The reaction is also pH independent at the pH's studied (5.7 and 7.4).

The ease with which NOB undergoes reduction to PHA in this study is in contrast to work by Dittmer and Kolyer (*23*), who found that NOB was reduced by 1-benzyl-1,4-dihydronicotinamide (an NADH analog) in refluxing ethanol to yield hydrazobenzene and aniline (the major product) in addition to PHA (no yield

reported). The reduction of NOB by NAD(P)H in the study described here occurred readily at 25°C in aqueous buffer and gave PHA as the sole product.

The chemical reduction of NOB to PHA takes on greater importance in connection with the suggestion that the *N*-arylhydroxylamines are necessary for the carcinogenic nature of the amine to be expressed (*1–3*). This study now shows that PHA can be readily generated from NOB by NAD(P)H in aqueous solution at ambient temperatures. Recent work by Corbett *et al.* (*24*) has shown that 4-chloroaniline in the presence of chloroperoxidase and hydrogen peroxide produces a single product, 4-chloronitrosobenzene. They point out that chloroperoxidase may be typical of oxidases which are quite abundant in many aquatic marine organisms, and that chlorinated anilines (common environmental residues generated from pesticides) may ultimately be transported to coastal marine environments and oxidized by such chloroperoxidases to arylnitroso compounds. In view of the results obtained in this study, it seems quite likely that with the availability of NAD(P)H the corresponding arylhydroxylamines may be generated from nitroso compounds even in the absence of an enzyme such as diaphorase. Because NADPH-mediated reduction of NOB follows a similar course in the presence and absence of enzyme (diaphorase), this reaction may also serve as a convenient model for studying enzyme catalysis.

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REFERENCES

1. J. A. MILLER AND E. C. MILLER, *Progr. Exp. Tumor Res.* **11,** 273 (1969).
2. F. F. KADLUBAR, J. A. MILLER, AND E. C. MILLER, *Cancer Res.* **36,** 1196 (1976).
3. E. KRIEK, *Biochim. Biophys. Acta* **355,** 117 (1974).
4. M. KIESE AND I. WIEDEMANN, *Biochem. Pharmacol.* **17,** 1151 (1968).
5. H. DANNENBERG AND M. KIESE, *Arch. Exp. Pathol. Pharmacol.* **211,** 102 (1950).
6. H. L. SCHMIDT, H. KEXEL, AND N. WEBER, *Biochem. Pharmacol.* **21,** 1641 (1972).
7. D. M. ZIEGLER, E. M. MCKEE, AND L. L. POULSEN, *Drug Metabol. Dispos.* **1,** 317 (1973).
8. F. F. KADLUBAR, J. A. MILLER, AND E. C. MILLER, *Cancer Res.* **36,** 2350 (1976).
9. E. C. MILLER AND J. A. MILLER, "The Molecular Biology of Cancer" (H. Busch, Ed.), pp. 377–398. Academic Press, New York, 1974 (and references therein).
10. E. C. MILLER, H. JUKL, AND J. A. MILLER, *Science* **153,** 1125 (1966).
11. E. E. SMISSMAN, AND M. D. CORBETT, *J. Org. Chem.* **37,** 1847 (1972).
12. R. E. THIERS, "Methods of Biochemical Analysis", Vol V, (D. Glick, Ed.), Vol. V, pp. 273–335. Interscience, New York, 1955. Metal-free buffer was used to avoid enhanced decomposition of PHA [Y. Ogata and T. Morimoto, *J. Org. Chem.* **30,** 597 (1965)].
13. M. GIBALDI AND D. PERRIER, "Pharmacokinetics," p. 281. Dekker, New York, 1975.
14. k_{NADH} or k_{NADPH} designates the value of k_r, using NADH or NADPH, respectively, as the reducing agent.
15. The appearance of nitrobenzene is in accord with the results from this laboratory on the study of the oxidation of PHA in aqueous buffer (T. KALHORN, A. R. BECKER, AND L. A. STERNSON, *Bio. Org. Chem.*, in press).

16. Studies from these laboratories indicate that azoxybenzene is also formed from the oxidation of PHA but its presence was not detectable using the hplc mobile phase conditions employed in this study.
17. H. DANNENBURG AND M. KIESE, *Arch. Exp. Pathol. Pharmakol.* **211,** 410 (1950).
18. M. KIESE, K. RESAG, AND C. SCHNEIDER, *Arch. Exp. Pathol. Pharmakol.* **231,** 170 (1957).
19. M. KIESE, H. UEHLEKE, AND N. WEGER, *Arch. Exp. Pathol. Pharmakol.* **242,** 130 (1961).
20. M. KIESE, AND H. D. WALLER, *Arch. Exp. Pathol. Pharmakol.* **211,** 345 (1950).
21. M. KIESE, D. REINWWIN, AND H. D. WALLER, *Arch. Exp. Pathol. Pharmakol.* **210,** 393 (1950).
22. M. L. C. BERHEIM, *Biochem. Biophys. Res. Commun.* **46,** 1598 (1972).
23. D. D. DITTMER AND J. M. KOLYER, *J. Org. Chem.* **27,** 56 (1962).
24. M. D. CORBETT, B. R. CHIPKO, AND D. G. BADEN, *Biochem. J.* **175,** 353 (1978).