[CONTRIBUTION FROM THE DEPARTMENT OF BACTERIOLOGY, UNIVERSITY OF WISCONSIN]

# Nitrous Oxide Inhibition of Nitrogen Fixation by *Azotobacter*[1]

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RECEIVED JANUARY 17, 1952

Specific inhibition of nitrogen fixation by nitrous oxide was confirmed by microrespiration studies with *Azotobacter vinelandii*. The type of inhibition was examined by using two partial pressures of nitrogen to each of which was added a $p_{N_2O}$ of 0.00, 0.25 and 0.50 atmosphere. Statistical analysis of the data revealed that nitrous oxide inhibition of nitrogen fixation in *Azotobacter* is competitive. The dissociation constant, $K_{N_2O}$, for the enzyme–inhibitor complex was estimated to be 0.08.

Hydrogen and carbon monoxide have been shown to inhibit nitrogen fixation in the symbiotic system of red clover and *Rhizobium* and in free living *Azotobacter*.[2-5] In both systems the inhibition by hydrogen is competitive, whereas inhibition by carbon monoxide is non-competitive. Because the ratio of enzyme–substrate dissociation constants of hydrogen and nitrogen approximated the ratio of their van der Waals constants, Molnar, *et al.*,[6] tested the hypothesis that inhibition by hydrogen could be attributed to physical adsorption by an examination of the effect of gases of widely different van der Waals constants. In the course of this study they discovered that another gas, nitrous oxide, is a specific inhibitor of nitrogen fixation by *Azotobacter*. Because of the significance of this finding for the chemical mechanism,[7] the present work was undertaken to determine the type of inhibition caused by nitrous oxide.

## Methods

*Azotobacter vinelandii* strain O was transferred daily on Burk nitrogen-free medium containing 2% sucrose. Cultures were incubated at 30° on a shaker; daily microscopic examination was made for purity. The test organisms for microrespiration studies were prepared by inoculating 50 ml. of medium with 1 ml. of a 24-hour culture. After 16–18 hours of incubation, this culture gave a 10–20 scale reading at 490 mμ on the Coleman spectrometer (optical density 1.000–0.699). An inoculum equivalent to 1 ml. at optical density 0.699 was added to 50 ml. of fresh medium and shaken vigorously to obtain an even suspension. Three ml. of this suspension was placed in each Warburg flask. Two-tenths ml. of 20% KOH and a strip of filter paper were placed in the center well.

Gas atmospheres were supplied in two series. In one series a $p_{N_2}$ of 0.10 atm. was used in combination with partial pressures of anesthesia $N_2O$ of 0.00, 0.25 and 0.50 atmospheres; in the second series the $p_{N_2}$ was raised to 0.30 atm., and the same partial pressures of nitrous oxide were added as in the first series. The $p_{O_2}$ of 0.20 atm. used in both series is considerably above the pressure at which oxygen is a limiting factor in nitrogen fixation. Helium was used as the inert "fill" gas to give a final pressure of 1 atmosphere. The source of nitrogen was air, tank oxygen being used to supplement the oxygen introduced with nitrogen.

A slight modification of the gassing procedure described by Umbreit, Burris and Stauffer[8] was used: instead of filling with a prepared gas mixture, each gas was introduced separately through a glass system that terminated in a capillary manifold. A bank of Warburg manometers was connected to the manifold outlets by capillary "h" shaped tubes. Before the addition of each gas to the flasks, the system leading to the flasks was evacuated to 0.1 atm. and filled with that gas; this procedure was repeated three times.

The water-bath was maintained at 30°. Five hourly readings were made during the course of the experiment. Oxygen consumed was replaced to maintain the $p_{O_2}$ at 0.2 atm.

## Results

When *Azotobacter* is in the logarithmic growth phase, the logarithm of oxygen uptake (μl. $O_2$ per hour.) plotted against time results in a straight line whose slope times 2.303 equals $k$, a first-order velocity constant for oxygen uptake, growth or nitrogen fixation (see Fig. 1). Wyss, *et al.*,[3] found that the rate of nitrogen fixation by *Azotobacter* varies as the $p_{N_2}$ is raised from 0.00 to approximately 0.3 atm. At this pressure near maximum fixation occurs. In our work the two partial pressures ($p_{N_2}$ 0.10 and 0.30 atm.) were chosen to permit evaluation of the effect of nitrous oxide on different rates of nitrogen fixation. Table I summarizes the results of six inhibition experiments; experiment I is graphically illustrated in Fig. 1. The slope of the lines determined by these points was calculated by the method of least squares. Control flasks with different partial

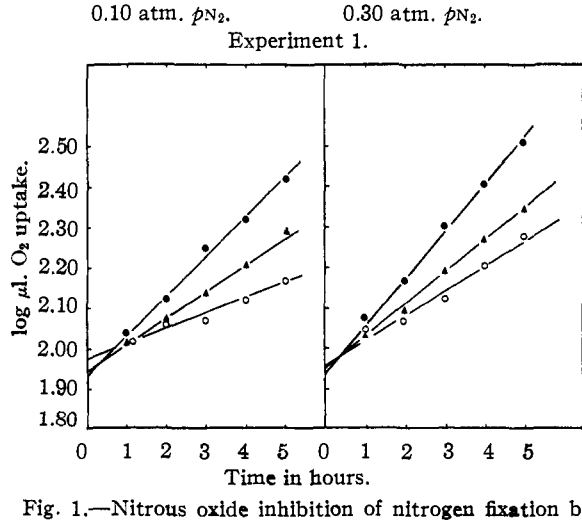


Fig. 1.—Nitrous oxide inhibition of nitrogen fixation by *Azotobacter vinelandii*: ●, 0.00 atm. $p_{N_2O}$; ▲, 0.25 atm. $p_{N_2O}$; ○, 0.50 atm. $p_{N_2O}$.

(1) Supported in part by grants from the Rockefeller Foundation, the Atomic Energy Commission, and the Research Committee of the Graduate School from funds provided by the Wisconsin Alumni Research Foundation.

(2) P. W. Wilson, S. B. Lee and O. Wyss, *J. Biol. Chem.*, **139**, 91 (1941).

(3) O. Wyss, C. J. Lind, J. B. Wilson, and P. W. Wilson, *Biochem. J.*, **35**, 845 (1941).

(4) C. J. Lind and P. W. Wilson, THIS JOURNAL, **63**, 3511 (1941).

(5) E. R. Ebersole, C. Guttentag and P. W. Wilson, *Arch. Biochem.*, **3**, 399 (1944).

(6) D. W. Molnar, R. H. Burris and P. W. Wilson, THIS JOURNAL, **70**, 1713 (1948).

(7) P. W. Wilson and R. H. Burris, *Bact. Revs.*, **11**, 41 (1947).

(8) W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques and Related Methods for the Study of Tissue Metabolism," second edition Burgess Publishing Co., Minneapolis, Minn. 1949, p. 45.

pressures of nitrogen had $k$ values of 0.222 and 0.265 for a $p_{N_2}$ of 0.10 and 0.30 atm., respectively. Increasing the $p_{N_2O}$ decreased the rate of nitrogen fixation as is evidenced by a decrease in the rate of oxygen uptake (Fig. 1). That this inhibition is specific for fixation of $N_2$ is demonstrated by the data from the two experiments presented in Table II. Independent of the gas atmospheres, the growth rates were not significantly different when 100 p.p.m. combined nitrogen as $(NH_4)_2HPO_4$-N was present.

TABLE I

EFFECT OF NITROUS OXIDE ON THE RATE OF NITROGEN FIXATION

| Expt. | $p_{N_2}$[a] | $p_{N_2O}$ | $k$[b] | $k/k_i$ | Expt. | $p_{N_2}$[a] | $p_{N_2O}$ | $k$[b] | $k/k_i$ |
|---|---|---|---|---|---|---|---|---|---|
| I | 0.10 | 0.00 | 0.222 | | IV | 0.10 | 0.00 | 0.286 | |
| | | .25 | .150 | 1.48 | | | .25 | .198 | 1.44 |
| | | .50 | .0834 | 2.66 | | | .50 | .176 | 1.62 |
| | .30 | .00 | .265 | | | .30 | .00 | .330 | |
| | | .25 | .183 | 1.45 | | | .25 | .253 | 1.30 |
| | | .50 | .140 | 1.89 | | | .50 | .213 | 1.55 |
| II[c] | .10 | .00 | .302 | | V | .10 | .00 | .320 | |
| | | .25 | .230 | 1.31 | | | .25 | .224 | 1.43 |
| | | .50 | .181 | 1.67 | | | .50 | .141 | 2.27 |
| | .30 | .00 | .320 | | | .30 | .00 | .327 | |
| | | .25 | .302 | 1.06 | | | .25 | .269 | 1.22 |
| | | .50 | .218 | 1.47 | | | .50 | .210 | 1.56 |
| III[c] | .10 | .00 | .316 | | VI | .10 | .00 | .235 | |
| | | .25 | .240 | 1.32 | | | .25 | .151 | 1.55 |
| | | .50 | .185 | 1.71 | | | .50 | .0839 | 2.80 |
| | .30 | .00 | .350 | | | .30 | .00 | .311 | |
| | | .25 | .276 | 1.27 | | | .25 | .258 | 1.21 |
| | | .50 | .211 | 1.66 | | | .50 | .160 | 1.94 |

[a] A $p_{O_2}$ of 0.20 atm. supplied to each gas mixture. Helium added as inert gas to give a final pressure of 1 atm. [b] $k$ value is first order velocity constant of nitrogen fixation. Each $k$ value determined by method of least squares from rates of duplicate flasks. [c] Four hourly readings made instead of five.

TABLE II

EFFECT OF $N_2O$ ON UPTAKE OF $NH_4^+$ BY *Azotobacter vinelandii*

| | Gas mixtures in atmospheres[a] | | | | | |
|---|---|---|---|---|---|---|
| $p_{N_2}$ | 0.10 | 0.10 | 0.10 | 0.30 | 0.30 | 0.30 |
| $p_{N_2O}$ | 0.00 | 0.25 | 0.50 | 0.00 | 0.25 | 0.50 |
| $k$ values with 100 p.p.m. $(NH_4)_2HPO_4$-N | 0.371 | 0.366 | 0.378 | 0.380 | 0.357 | 0.348 |
| $k$ values without $(NH_4)_2HPO_4$-N | .339 | .359 | .352 | .369 | .348 | .354 |
| | .258 | | | .332 | | |
| | .232 | | | .295 | | |

[a] A $p_{O_2}$ of 0.2 was supplied in all gas mixtures; helium was used to give a final pressure of 1 atmosphere.

The type of inhibition can be determined by applying the equations of Lineweaver and Burk as described by Ebersole, *et al.*[5]

$$\frac{v}{v_i} \text{ or } \frac{k}{k_i} = 1 + \frac{K_s}{K_i}\frac{(I)}{K_s + (S)}$$

$k/k_i$ represents the relative velocity of the reaction, $K_i$ the dissociation constant of enzyme–inhibitor complex, $K_s$ the dissociation constant of the enzyme–substrate complex, (I) is the concentration of the inhibitor, and (S) is the concentration of the substrate. This formula states that if $k/k_i$ is plotted against different concentrations of inhibitor ($N_2O$) for two concentrations of substrate ($N_2$), lines should be formed with unit intercepts. If the resulting lines have significantly different slopes, inhibition is competitive; if the lines coincide, inhibition is non-competitive.

The statistical method used to determine these slopes from points obtained from several experiments has been discussed by Wilson, *et al.*[2] Briefly, for a line with an intercept of unity the slope, $b$, is calculated from

$$b = \frac{\Sigma xy - \Sigma x}{\Sigma x^2}$$

where $x$, the independent variable, equals the concentration of the inhibitor, and $y$, the dependent variable, equals $k/k_i$. The difference between any two slopes, $b_1 - b_2$, is tested by calculation of the statistic $t$; in which $S^2$ is the variance of $b$ calculated from $N$ paired observations of $x$ and $y$.[9]

$$t = \frac{b_1 - b_2}{\sqrt{\frac{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2}{N_1 + N_2 - 2}\left[\frac{1}{\Sigma x_1^2} + \frac{1}{\Sigma x_2^2}\right]}}$$

If the variances are homogeneous, the calculated $t$ value is compared with a theoretical value of $t_{0.05}$ (19 to 1 odds) for $N_1 + N_2 - 2$ degrees of freedom. If the variances are not homogeneous, the calculated $t$ value can no longer be compared with a specific theoretical value of $t_{0.05}$ but only with a range. The limits of this range are the values of $t_{0.05}$ for $N_1 - 1$ and $N_1 + N_2 - 2$ degrees of freedom. Only when the experimental value of $t$ falls outside the limits of the range does an unequivocal conclusion become possible.

The relevant statistics estimated from Experiments I–VI are summarized in Table III. The observed difference between the slopes was demonstrated to be statistically significant. Although neither variance is excessive, they are not statistically homogeneous. The range of $t_{0.05}$ for 11 and 22 degrees of freedom is 2.201 and 2.074. As the experimental $t$ (3.396) exceeds both values, it is concluded that nitrous oxide competitively inhibits nitrogen fixation by *Azotobacter*.

TABLE III

STATISTICAL SUMMARY OF INHIBITION DATA

| $p_{N_2}$[a] | N[b] | $b$[c] | Variance of $b$ | $t_{0.05}$ $N_1 - 1$ | $t_{0.05}$ $N_1 + N_2 - 2$ |
|---|---|---|---|---|---|
| 0.10 | 12 | 2.1427 | 0.2320 | | |
| 0.30 | 12 | 1.2880 | 0.0277 | | |
| | | | | 3.396 | 2.201 2.074 |

[a] In atmospheres. [b] Number of paired observations. [c] Calculated slope of line when $k/k_i$ is plotted against $p_{N_2O}$.

The data obtained also permit an estimation of the dissociation constant $K_{N_2O}$ for the enzyme–nitrous oxide complex. Methods for obtaining this value are given by Wilson, *et al.*[10] They found the dissociation constant $K_{N_2}$ of enzyme–nitrogen complex to be 0.02 ± 0.005 atmosphere, and the dissociation constant $K_{H_2}$ for enzyme inhibitor to be 0.11 ± 0.028 atm. In the present work the enzyme–nitrous oxide complex dissociation constant $K_{N_2O}$ was found to be 0.08 atmosphere.

## Discussion

Wilson and Burris[7] have discussed three schemes as possible mechanisms of biological nitrogen fixation. The first two employ direct reduction of molecular nitrogen to $NH_4^+$; the third involves oxidation of molecular nitrogen before its ultimate

(9) C. Eisenhart and P. W. Wilson, *Bact. Revs.*, **7**, 57 (1943).

(10) P. W. Wilson, R. H. Burris and C. J. Lind, *Proc. Natl. Acad. Sci., U. S.*, **28**, 243 (1942).

reduction to ammonia. If an oxidation does occur, a logical intermediate would be hyponitrite, the generally accepted intermediate in the reduction of nitrates to ammonia and nitrogen. The experimental data presented here suggesting that nitrous oxide is a competitive inhibitor of the nitrogen fixation reaction in *Azotobacter* can be interpreted as some support for this view.[11]

It is assumed that nitrous oxide affects some reaction prior to formation of ammonia, because inhibition cannot be demonstrated in the presence of this compound (Table II). These initial steps in the fixation process have escaped detection, and the enzyme or enzymes involved are not known. Nitrous oxide apparently competes for an enzyme active in this reaction. If nitrous oxide or a related compound were an intermediate in the nitrogen fixation reaction, one would anticipate not only competitive inhibition but also ready utilization. Since nitrous oxide is not assimilated by *Azotobacter* the most likely present explanation is that nitrous oxide has properties similar enough to some intermediate to enable it to compete for the enzyme—*i.e.*, analogous to the action of an antimetabolite. If nitrous oxide has the same effect on the symbiotic nitrogen fixing system as on that of *Azotobacter*, Kriegel's discovery confirmed by Taylor, *et al.*,[12] that nitrous oxide is one of the most abundant constituents of soil air could have important practical implications for agriculture.

(11) In a note published in *Chemistry and Industry*, no. 4, 87 (1952), T. G. G. Wilson and E. R. Roberts report independent confirmation of this important conclusion.

(12) R. C. Taylor, R. A. Brown, W. S. Young and C. E. Headington, *Anal. Chem.*, **20**, 396 (1948).

MADISON, WISCONSIN

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[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT OF THE UNIVERSITY OF LOUISVILLE]

# Polarography of Some Substituted 8-Quinolinols

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The polarographic behavior of 2-, 3- and 4-methyl substituted 8-quinolinols in the *p*H range 2–12 was compared with that of 8-quinolinol. The 3-methyl derivative resembled 8-quinolinol much more closely than the other compounds. The addition of aluminum ion had no effect on the waves of 2-methyl- and 2,3-dimethyl-8-quinolinol in alkaline solution, indicating no chelate formation.

As part of a study of the properties of substituted 8-quinolinols[2a,b,3] the polarography of the 2-, 3- and 4-methyl and 2,3-dimethyl derivatives was studied. In spite of the close structural relationship of these compounds differences in reduction at the mercury cathode were anticipated from the greater activities of 2- and 4-substituents compared to 3-substituents in quinolines, and also from the anomalous behavior of 2-substituted 8-quinolinols in forming no chelates with aluminum ion.[2a,4,5]

## Experimental

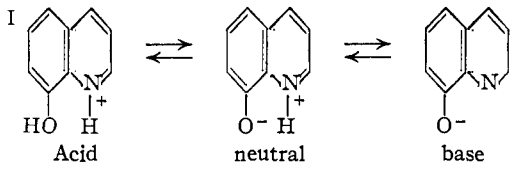
A Sargent Model XXI Polarograph and an H-type cell kept at 25 ± 0.01° were employed for all determinations. Measurements were made against a saturated calomel electrode. The characteristics of the dropping mercury electrode, determined in 0.1 *N* potassium chloride on open circuit, were: $m = 2.30$ mg./sec., $t = 4.00$ sec.

Polarograms were run on each compound at nine or more *p*H values between 2 and 12 and at concentrations ranging from 0.0001 to 0.001 *M*. Britton and Robinson buffers (consisting of acetic, phosphoric and boric acids with sodium hydroxide) were used after polarographic examination for reducible impurities. Oxygen was removed from the solutions with nitrogen. An instrument sensitivity of 0.100 was usually suitable.

The preparation and purification of the compounds have been previously described.[2a]

## Results and Discussion

The polarographic reduction of substituted 8-quinolinols shows great variations with *p*H. It is convenient to consider the polarograms in acidic, neutral and basic solutions separately since there are three different molecular species involved




In acid solutions the reduction of 8-quinolinol and presumably of its methyl derivatives, is known to give the 1,2,3,4-tetrahydro derivative.[6,7] Polarographically it is not possible to establish the reduction process because of a catalytic wave due to the quinolinium ion.[8] The magnitude of the catalytic wave, as well as its potential, decreases with increasing *p*H (Figs. 1–3), because an increase in *p*H shifts the equilibrium of equation I to give decreasing amounts of quinolinium ion in solution. In all the compounds a small preliminary wave at the foot of the catalytic wave was noticeable but the interference of the following catalytic wave made analysis impossible. The preliminary wave was most pronounced in 2,3-dimethyl-8-quinolinol.

In neutral solutions from *p*H 4 to 9.5 the analysis of the polarographic waves is complicated by the unknown position of the equilibria of equation I. The waves in this portion of the *p*H range showed greater variations from compound to compound than anywhere else. With all compounds pronounced maxima were obtained at a potential of

(1) International Fellow from Colombo, Ceylon, sponsored by Joseph E. Seagram and Sons, Inc., Louisville, Kentucky.

(2) (a) J. P. Phillips and L. L. Merritt, THIS JOURNAL, **71**, 3984 (1949); (b) J. P. Phillips and F. J. O'Hara, *ibid.*, **73**, 583 (1951).

(3) J. P. Phillips and H. P. Price, *ibid.*, **73**, 4414 (1951).

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(8) S. Wawzonek, *Anal. Chem.*, **21**, 61 (1949).