constant 34.5 can only be due to interaction between the ions (in the ion pairs) with the acetone molecules, on the one hand, and lack of such interaction with nitrobenzene molecules, on the other. A comparison of sodium and potassium picrates in acetone and nitrobenzene shows that these salts behave in a manner similar to that of lithium picrate. The Walden conductance viscosity product for the sodium and potassium ions in acetone is 0.238 and 0.242, respectively, while in nitrobenzene[11] it is 0.295 and 0.322. Thus, the free ions in this solvent are of very nearly the same size. The corresponding K values $\times 10^4$ are, respectively, 13.5 and 34.3 in acetone and 0.28 and 6.86 in nitrobenzene. The effective size of these ions in the ion pairs is much smaller in nitrobenzene than in acetone and is progressively more so as the (lattice) ion is smaller.

V. Summary

A simplified procedure for the purification of acetone has been developed, making use of activated alumina pellets.

The density, viscosity and dielectric constant of purified acetone at 25° have been determined.

Conductance data have been obtained for fourteen different salts in acetone solution at 25°.

Limiting conductances and dissociation constants have been calculated for these salts, using the extrapolation method of Fuoss.

Limiting ion conductances have been computed by the method of Fowler.

Anion conductances have been found to be, in general, greater than corresponding cation conductances, suggesting specific solvent interaction with cations.

The conductance of the fluoride ion has been found to be markedly lower than that of the other halide ions and abnormally low for an anion.

PROVIDENCE, R. I. RECEIVED JANUARY 14, 1948

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND THE DEPARTMENT OF AGRICULTURAL BACTERIOLOGY UNIVERSITY OF WISCONSIN]

The Effect of Various Gases on Nitrogen Fixation by *Azotobacter*[1]

BY DOROTHY M. MOLNAR, R. H. BURRIS AND P. W. WILSON

Molecular hydrogen was demonstrated by Wilson and his associates[2,3,4] to inhibit nitrogen fixation, specifically and competitively, in both free-living *Azotobacter* and the symbiotic system of red clover plus *Rhizobium*. It has been suggested[5] that the inhibition might be primarily a physical effect dependent on the relative adsorption of the two gases on the surface of the nitrogen-fixing enzyme. The observation that the enzyme-substrate dissociation constants of hydrogen and nitrogen in *Azotobacter* have essentially the same ratio as their van der Waals constants offers some support for this view.[6] Although this may be only fortuitous, an examination of the effect of gases with different physical properties, appears to be desirable.

Experimental

Methods.—Cultures of *Azotobacter vinelandii* were maintained by daily transfer to 50 ml. of Burk's medium[3] in 500-ml. Erlenmeyer "shake" flasks. Weekly tests for purity were made by microscopic examination (Gram stain) and by inoculation of beef extract–peptone broth.[5] For the microrespirometer studies, ten drops of a culture (seventeen to nineteen hours) was diluted with 40 ml. of sterile Burk's medium, and 2 ml. transferred to the respiration flask. The conventional techniques for supplying different gas mixtures in respiratory experiments were followed.[7] Manometer readings were taken at half-hour intervals over a period of five hours, at 30°. The gas mixtures were: p_{N_2}, 0.2 atm.; p_{O_2}, 0.2 atm.; helium, argon, neon, hydrogen, ethane, or nitrous oxide, 0.6 atm. At a partial pressure of nitrogen of 0.2 atm. the rate of fixation is about 95% of maximum (in the absence of a competitive inhibitor, such as H_2), and small variations in the pressure (± 0.02 atm.) cause little change. The partial pressure of oxygen likewise is near optimum; to insure that is was kept reasonably constant, the oxygen used in respiration was replaced periodically. The mixtures were made from ordinary cylinder gases (about 98–99% pure) with the exception of the nitrous oxide, which was the grade used for anaesthesia. Errors arising from variation in the composition were reduced by preparing separate mixtures for the replicate experiments.

In a few trials, the conclusions from the microrespiration data were checked by estimating the initial and final total nitrogen in representative flasks by a modification of the micromethod of Johnson.[8] One macro experiment was made: Ten ml. of an eighteen-hour culture was diluted with 300 ml. of sterile Burk's medium, and 25-ml. aliquots were pipetted into sterile 250 ml. Erlenmeyer flasks. Each flask contained a tube of potash to absorb respiratory carbon dioxide and was made gas-tight with a rubber stopper fitted with an inlet tube. After the desired gas mixture was supplied through the inlet tube, the flasks were incubated in a conventional shaking apparatus at 30° for fifteen hours, then total nitrogen determined on aliquots by the micromethod.

Results and Discussion

The van der Waals constant a for the gases tested varied from 0.00007 (helium) to 0.01074

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

(2) Wilson, "The Biochemistry of Symbiotic Nitrogen Fixation," University of Wisconsin Press, Madison, Wisconsin, 1940.

(3) Wyss and Wilson, *Proc. Natl. Acad. Sci. (U. S.)*, **27**, 162 (1941).

(4) Wyss, Lind, Wilson and Wilson, *Biochem. J.*, **35**, 845 (1941).

(5) Burk and Burris, *Ann. Rev. Biochem.*, **10**, 587 (1941).

(6) Wilson and Burris, *Bact. Rev.*, **11**, 41 (1947).

(7) Umbreit, Burris and Stauffer, "Manometric Techniques and Related Methods for the Study of Tissue Metabolism," Burgess Publishing Co., Minneapolis, Minn., 1945.

(8) Johnson, *J. Biol. Chem.*, **137**, 575 (1941).

(ethane); a wide range in physical properties such as solubility, adsorption on solids, boiling point, are known to be associated with this variation. If the physiological response of *Azotobacter* to hydrogen is to be ascribed to the physical characteristics of the gases in the mixture supplied as expressed in the van der Waals forces, one should expect some correlation of nitrogen fixation with the value of a of the diluent gas. The data in Tables I, II and III and in Fig. 1 demonstrate that there is no such correlation.

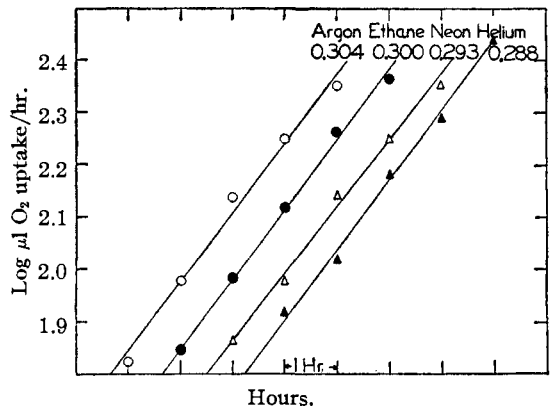


Fig. 1.—Effect of different gases on nitrogen fixation by *Azotobacter vinelandii*. Points are the means of duplicates for ethane, of triplicates for the others. Each division of the abscissa in Figs. 1 and 2 is one hour.

Table I summarizes results from two representative experiments. The initial and final rates of respiration are included to illustrate the effect of the gas tested on final total nitrogen as measured by rate of respiration. But, as has been emphasized in the previous papers,[3,4] a more suitable criterion of the effect is given by the first order velocity constant of growth, k. The values of k were estimated from the slopes of the lines obtained when the rate of respiration was plotted as a function of time. The standard deviation of k, S_k, was calculated by the usual statistical procedure; it measures the departure of the points from a straight line and is in effect an estimate of the precision of the data. The slope of each line, and hence k, was calculated by the method of least squares. The variation in the k values of the replicate samples measures the reproducibility of the results. Considering these variations, together with the observed values of S_k, it is concluded that for single determinations differences of 10–20% are necessary for significance, but if 2–3 replicates are combined, differences of 5–10% are probably significant. The precision obtained when replicate samples are combined is illustrated graphically by the lines in Fig. 1.

Table II summarizes the results of all the microrespiration experiments. For convenience, the van der Waals constant, a, is included, together with the boiling point of the gases, a physical property known to be correlated with a. In each experiment a helium control was included since it has been previously demonstrated that this gas does not affect fixation; this point was checked occasionally by including an air control. It is evident from the table that the physiological function of nitrogen fixation in *Azotobacter* shows no such response to changes in the van der Waals forces of the diluent gas as does a typical physical quantity (b. p.). Explanation of inhibition of nitrogen fixation in *Azotobacter* by a hypothesis based on purely physical competition between hydrogen and nitrogen, therefore, appears unlikely. The

TABLE I

EFFECT OF VARIOUS GASES ON NITROGEN FIXATION BY *Azotobacter* (MICRORESPIRATION DATA)

Gas[a]	Rate of respiration[b] Initial	Final	k[c]	S_k
		Experiment I		
Nitrous oxide	78	117	0.108	0.006
	74	136	.163	.004
	67	139	.196	.010
	80	140	.145	.004
Helium	82	245	.271	.015
	90	255	.262	.007
	83	263	.283	.012
Ethane	81	226	.246	.019
	85	260	.269	.023
	77	265	.301	.021
Air	90	258	.267	.011
	103	288	.267	.018
		Experiment II		
Helium	91	310	0.285	0.015
	86	318	.306	.008
	90	286	.274	.004
Ethane	79	270	.292	.003
	80	286	.308	.019
Neon	75	289	.311	.019
	80	269	.285	.015
	77	256	.271	.013
Argon	72	283	.306	.016
	73	275	.306	.019
	67	306	.301	.023

[a] Atmosphere: 20% N_2, 20% O_2, 60% indicated gas. [b] Microliters O_2/hr./flask, at 30°. [c] First order velocity constant of fixation; S_k, its standard deviation, measures closeness of fit of points to line.

TABLE II

SUMMARY OF THE MICRORESPIRATION EXPERIMENTS

Gas	van der Waals $a \times 10^5$	B. p., °C.	Expts.	Inhibition of N fixation[a]
Ethane	1074	88.3	3	−6.4 ± 4.5
Nitrous oxide	754	89.5	10	45.0 ± 3.9
Nitrogen	277	195.8	..	.....
Argon	268	185.7	3	−1.6 ± 3.1
Neon	42	245.9	3	1.8 ± 4.0
Hydrogen	49	252.8	5	35.2 ± 7.3
Helium	7	268.9	..	.....

[a] Based on helium control: negative results indicate stimulation.

physiological explanation,[9] based on enzyme mechanisms, thus receives indirect support.

An important by-product of these experiments was the demonstration that nitrous oxide is a specific inhibitor of nitrogen fixation by *Azotobacter vinelandii*. Because of the possible implication of this discovery for the mechanism of fixation, ten separate experiments were made to establish this finding. Inhibition was obtained in every trial, ranging from 21 to 63% with an average of 45 ± 3.9%. Confirmation of the data from the microrespiration experiments by determining the actual quantities of nitrogen fixed is furnished by the results given in Table III.

TABLE III

EFFECT OF VARIOUS GASES ON NITROGEN FIXATION BY *Azotobacter* (TOTAL NITROGEN DATA)

Experiment[a]	Diluent gas	Time, hr.	Final total N γ/ml.
III	He		28.88
(8.75)	N_2O	7	17.50
	H_2		20.38
IV	He		23.38
(8.33)	N_2O	7	15.69
	H_2		15.00
V	He	7	19.60
(5.60)	N_2O		10.00
VI	He	15	48.5
(5.20)			46.0
	A		42.6
			50.6
	N_2O		17.4
			15.4
			23.4
			14.8

[a] Figures in parentheses in this column represent initial nitrogen content in γ/ml. Experiment VI is a macro experiment; the others are samples taken from microrespiration experiments.

That the inhibition by nitrous oxide is specific for nitrogen fixation as distinguished from assimilation of combined nitrogen is shown by the results in Table IV and in Fig. 2. Assimilation of ammonium apparently is slightly stimulated by nitrous oxide in the atmosphere, but as this effect is not consistently obtained in all trials, its establishment would require much more additional evidence.

Previously, only hydrogen and carbon monoxide were known to influence the fixation reaction *specifically* in *Azotobacter*. Not only does the finding that nitrous oxide is likewise a specific inhibitor provide a new tool for investigation but it may also possess special significance for the mechanism. In a recent review Wilson and Burris[6] suggested that a possible intermediate in the fixation reaction might be a compound that is formally analogous to hyponitrous acid though not necessarily identical with it. The fact that nitrous oxide, the anhydride of hyponitrous acid, is not utilized by

(9) Lee and Wilson, *J. Biol. Chem.*, **151**, 377 (1943).

TABLE IV

COMPARISON OF EFFECT OF NITROUS OXIDE ON ASSIMILATION OF FREE AND COMBINED NITROGEN BY *Azotobacter* (MICRORESPIRATION DATA)

Expt.	Source of N	Gas added (0.6 atm.)[a] He	H_2	N_2O
VII	N_2	0.361	0.124	0.150
		.361	.143	.116
	NH_4^+	.319	.299	.325
		.323	.304	.323
VIII	N_2	.278	.194	.138
		.320	.199	.140
	NH_4^+	.327	.300	.375
		.317	.340	.394
IX	N_2	.290	.228	.182
		.297	.221	.143
	NH_4^+	.306	.350	.320
		.306	.331	.320

[a] Data are *k* values calculated from lines.

Azotobacter but does interfere with the assimilation of molecular nitrogen could be interpreted as supporting evidence for this view. Final decision as to its significance must await a more detailed study, particularly whether the inhibition is competitive or non-competitive.

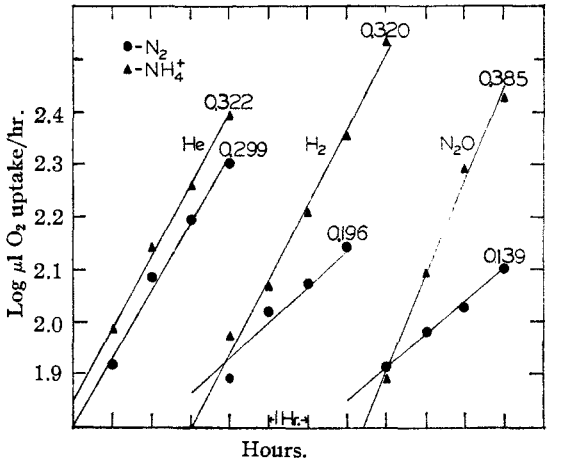


Fig. 2.—Specific inhibition of nitrogen fixation by *Azotobacter vinelandii*. Each point is the mean of duplicates.

Summary

The hypothesis that the inhibition by molecular hydrogen of nitrogen fixation in *Azotobacter* is explicable on the basis of the physical properties of the two gases was tested by comparing the effects of helium, argon, neon, hydrogen, nitrous oxide and ethane on the fixation reaction. The microrespiration technique was used for most of the experiments, but the results were verified by total nitrogen determinations.

The van der Waals constant, *a*, of the gases tested ranged from 0.00007 to 0.01074. Although the physical properties of the gases are correlated with the van der Waals forces, no such correlation

appeared in their effect on the physiological function of nitrogen fixation in *Azotobacter*. It is concluded, therefore, that an explanation based on the relative physical properties of hydrogen and nitrogen is unlikely.

Nitrous oxide was found to be a specific inhibitor for nitrogen fixation by *Azotobacter*. Its inhibition may have important implications for the mechanism of the reaction.

MADISON 6, WISCONSIN RECEIVED DECEMBER 9, 1947

[CONTRIBUTION FROM THE INSTITUTE OF EXPERIMENTAL BIOLOGY, UNIVERSITY OF CALIFORNIA]

Kinetics of the Reactions between Iodine and Certain Substituted Phenols

BY CHOH HAO LI

In previous studies[1] it was shown that the reaction of tyrosine with iodine follows a bimolecular rate law and that the most reactive iodinating agent is hypoiodous acid. It was also demonstrated[2] that the formation of diiodotyrosine is catalyzed by phosphate and other basic ions. The present investigation extends such studies with other para substituted phenols.

Reactions were carried out at 25° in acetate buffers of *p*H 5.23 and 5.65 containing different iodide ion concentrations. *p*-Chlorophenol and *p*-hydroxyphenylethylamine (tyramine), C. P. crystalline preparations, were employed without further purification; glycyl-tyrosine was kindly supplied by Dr. J. S. Fruton and the late Dr. Max Bergmann. The reaction rates were followed in the manner previously[1] described. The rate law was found to be identical with that for the formation of diiodotyrosine and may be expressed by the equation

$$-\,\mathrm{d(Phenol)}/\mathrm{d}t = k_2(\mathrm{phenol})(\mathrm{I_3^-}) \qquad (1)$$

where k_2 is the specific rate constant for the reaction Phenol + $2I_2 \rightarrow$ diiodophenol + $2H^+$ + $2I^-$

Table I summarizes the specific rate constants for the formation of diiodophenols[3]; the values for the reaction between iodine and tyrosine were estimated from previous studies.[1] The concentrations are in moles per liter, and time in minutes. It may be noted that the product of $k_2(I^-)^2$ in each buffer appears to be rather constant at the range of iodide concentration studied. In concentrations of iodide beyond the range studied, $k_2(I^-)^2$ was not found to be constant.

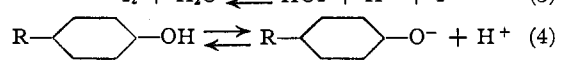
In the study of diiodotyrosine formation,[1] it was found that the reaction involves four paths: namely, iodine and phenol, iodine and phenolate, hypoiodous acid and phenol, and hypoiodous acid and phenolate. It was further noted that the most reactive pair is hypoiodous acid and phenolate, whereas the reaction between iodine and phenol is the least reactive. For first approximations, the reaction between iodine and phenol may be represented by equation (1a).

$$-\mathrm{d(phenol)}/\mathrm{d}t = k'(\mathrm{phenol})(\mathrm{HOI}) + k''(\mathrm{phenolate})(\mathrm{HOI}) \qquad (1a)$$

From the equilibria

$$\mathrm{I^-_3} \rightleftarrows \mathrm{I_2} + \mathrm{I^-} \qquad (2)$$

$$\mathrm{I_2} + \mathrm{H_2O} \rightleftarrows \mathrm{HOI} + \mathrm{H^+} + \mathrm{I^-} \qquad (3)$$



(4)

TABLE I

SPECIFIC REACTION RATE, k_2, OF IODINATING *p*-CHLOROPHENOL, GLYCYLTYROSINE, TYRAMINE AND TYROSINE[a] IN ACETATE BUFFERS OF *p*H 5.23 AND *p*H 5.65 CONTAINING DIFFERENT IODIDE CONCENTRATION AT 25°

k_2 in gram moles per liter per minute

(I^-) $m \times 10^2$	*p*-Chlorophenol		Tyramine		Glycyl-tyrosine		Tyrosine	
	*p*H 5.23	*p*H 5.65	*p*H 5.23	*p*H 5.65	*p*H 5.23	*p*H 5.65	*p*H 5.23	*p*H 5.65
3.34	0.135(0.128)[b]	0.40(0.34)	0.70(0.72)	2.03(2.29)	0.91(0.94)	3.15(3.21)	0.52(0.53)	1.82(1.94)
4.08	.081(.085)	.26(.26)	.45(.45)	1.63(1.49)	.63(.63)	2.16(2.14)	.38(.36)	1.20(1.30)
4.84	.060(.061)	.18(.18)	.35(.38)	1.09(1.07)	.45(.49)	1.55(1.55)	.28(.26)	0.90(0.93)
6.34	.033(.035)	.11(.11)	.20(.22)	0.63(0.62)			.16(.15)	0.53(0.54)

[a] Reaction rates for tyrosine are from a previous paper (see ref. 1). [b] The figures in parentheses are computed values from Equations 7, 8, 9 and 10.

(1) Li, THIS JOURNAL, **64**, 1147 (1942).

(2) Li, *ibid.*, **66**, 228 (1944).

(3) The preparation of N-glycyl-3,5-diiodotyrosine has been reported by Abderhalden and Guggenheim [*Ber.*, **41**, 1241 (1908)]. Diiodotyramine has also been prepared by Abderhalden and his coworkers [*Arch. ges. Physiol.*, **195**, 167 (1922)]. As far as we are aware there is no report concerning the preparation of *p*-chloro-3,5-diiodophenol. In the present experiments we have observed that *p*-chloro-3,5-diiodophenol is very insoluble in acetate buffers and it is gradually crystallized out in fine needles as the reaction proceeds. The colorless crystals have a melting point at 108.5°. *Anal.* Calcd. for $HOC_6H_2I_2Cl$: I, 66.74. Found: I, 66.82.

Equation (1a) becomes

$$-\frac{\mathrm{d(phenol)}}{\mathrm{d}t} = \frac{K_2}{(\mathrm{I^-})^2(\mathrm{H^+})}\left[k'K_3 + \frac{k''K_3K_4}{(\mathrm{H^+})}\right](\mathrm{phenol})(\mathrm{I_3^-}) \qquad (5)$$

where K_2, K_3 and K_4 are the equilibrium constants of equations 2, 3, and 4, respectively. Thus, by comparing the equations (1) and (5), the observed specific rate constant, k_2, is a function of iodide and