ACTION OF INHIBITORS ON HYDROGENASE IN AZOTOBACTER*

By J. B. Wilson and P. W. Wilson

(*From the Department of Agricultural Bacteriology, University of Wisconsin, Madison*)

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INTRODUCTION

Previous studies on the occurrence and properties of hydrogenase in *Azotobacter vinelandii* have included a few tests with typical respiratory enzyme inhibitors, but a detailed survey of the effect of inhibitors on this enzyme has not been made. Such investigations are desirable for the following specific purposes:—

1. To provide basic information concerning the nature of the enzyme system. This is essential not only for its intrinsic value but in order to compare the hydrogenase in *Azotobacter* with that in other species.

2. To compare the properties of the enzyme as it functions in the intact cell and in cell-free extracts. Because H_2 is such a specific substrate its oxidation by a suspension of *Azotobacter* can be readily measured with a minimum of complications arising from concurrent oxidations of other substrates. This *Knallgas* reaction is also catalyzed by cell-free enzyme preparations (Lee, Wilson, and Wilson, 1942). An opportunity is thus furnished to test with an oxidative enzyme system the constantly disputed question as to whether results obtained with cell-free enzymes are applicable to biochemical reactions in the intact cell.

3. To discover, if possible, an inhibitor with differential effects on the oxidation of hydrogen and of other substrates. Although concomitant respiratory activity is not a serious difficulty in studies with azotobacter, it becomes so with suspensions of the root nodule bacteria (*Rhizobium*, sp.) taken directly from the nodules of leguminous plants. Such suspensions retain a high "endogenous" respiration in spite of repeated washings (Thorne and Burris, 1940). For this reason demonstration of a hydrogenase is difficult. Because of its significance for the mechanism of fixation (Lee, Wilson, and Wilson, 1942), more extensive information respecting the occurrence of hydrogenase in nodule suspensions should be secured.

Methods

Details of the method for measuring hydrogenase activity in cultures of *Azotobacter vinelandii* have been described by Wilson, Lee, and Wilson (1942).

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Essentially, the gas uptake in a H_2-O_2 mixture by a "resting" suspension which contains about 0.1 mg. N is measured at 35°C. in a Warburg microrespirometer. The rate, which remains constant over the period of the run (about 60 minutes), is calculated as the Q_K (N) = c.mm. total gas uptake per hour per mg. cell N. A suitable control on respiration is made in air or in a He-O_2 mixture, and the rate calculated as the Q_{O_2} (N) = c.mm. O_2 uptake per hour per mg. cell N.

EXPERIMENTAL

Action of Inhibitors on Oxidation of H_2 and Other Substrates by Azotobacter

Cyanide.—Lee *et al.* (1942) reported that M/1000 KCN at pH 7.5 completely inhibited the hydrogenase in *Azotobacter*. This has been confirmed in two

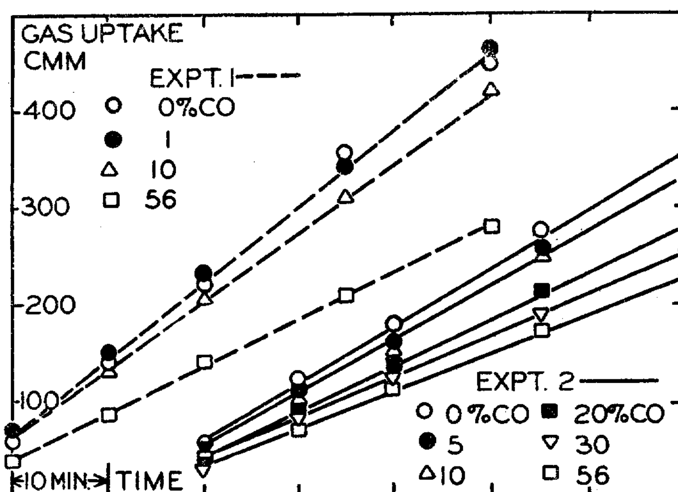

FIG. 1. Effect of CO on oxidation of hydrogen by *Azotobacter vinelandii*. Gas mixture: pH_2, 0.4; pO_2, 0.04 atm.; indicated CO and helium to 1 atm.

additional experiments indicating that the *Knallgas* reaction is entirely cyanide-sensitive.

Carbon Monoxide.—The oxidation of hydrogen by *Azotobacter* is likewise very sensitive to CO (Fig. 1). The composition of the gas mixtures in these experiments was 4 per cent O_2, 40 per cent H_2, the indicated quantity of CO, and He to one atmosphere. A definite effect on the rate of hydrogen oxidation is observed with as little as 5 to 10 per cent CO, and with 56 per cent CO the rate may be inhibited as much as 50 per cent. In Experiment 1 the Q_K(N) values for different ratios of CO/O_2 (given in parentheses) were: (0), 4900; (1/4), 4900; (5/2), 4500; (14/1), 3300. In Experiment 2 these were: (0), 5140; (5/4), 4750; (5/2), 4720; (5/1), 4000; (15/2), 3430; (14/1), 3140. Controls in which helium was substituted for H_2 demonstrated that respiration on

glucose or succinate was inhibited slightly, if at all, even with 56 per cent CO in the gas mixture.

Attempts to reverse the CO inhibition with light were unsuccessful. Recently, Dr. H. D. Hoberman informed us in a private communication that he had observed a similar CO inhibition which was light reversible with a hydrogenase in *Proteus vulgaris*. Other information from Dr. Hoberman indicated that the enzyme in *Proteus* is similar in many of its properties to that

TABLE I

Inhibition of Respiratory and Hydrogenase Activity in Azotobacter vinelandii by Sodium Azide

Experiment No.	Concentration of azide	Respiration		Hydrogenase	
		$Q_{O_2}(N)$	Inhibition	$Q_K(N)$	Inhibition
1	None	—	—	3200	—
	M/100	—	—	2200	31
2	None	—	—	3330	—
	M/100	—	—	2530	24
3	None	3560	—	4320	—
	M/4500	3280	8	4320	0
	M/900	2630	26	4540	−5
	M/450	1190	66	4307	0
	M/90	1380	71	3380	22
	M/9	57	98	1077	75
4	None	4200	—	2100	—
	M/10,000	4200	0	—	
	M/5000	4250	−1	3160	−50
	M/1000	3320	21	3020	−44
	M/500	2440	42	2660	−27
	M/100	430	89	1410	33
	M/10	113	97	300	86

Respiration: M/150 sucrose in air.
Hydrogenase: no added substrate in 96 per cent H_2, 4 per cent O_2.

in *Azotobacter*. It appears likely, then, that the CO inhibition of hydrogenase in *Azotobacter* is also light-sensitive. Additional experiments are in progress to investigate this point in detail.

Sodium Azide.—Lee *et al.* (1942) found that sodium azide at its optimum pH (6.5) was less inhibitory than cyanide. This has been verified in further trials as illustrated by the data in Table I. With low concentrations of azide stimulation is frequently observed; at M/100 there is an inhibition of about 25 per cent which increases to 75 to 85 per cent at M/10. A marked differential inhibition of the oxidation of H_2 and other substrates is obtained in the range M/1000 to M/100.

Hydroxylamine.—Data in Fig. 2 and Table II demonstrate that hydroxylamine has a pronounced differential inhibitory effect on the respiration and hydrogenase activity of *A. vinelandii.* Whereas respiration on glucose and

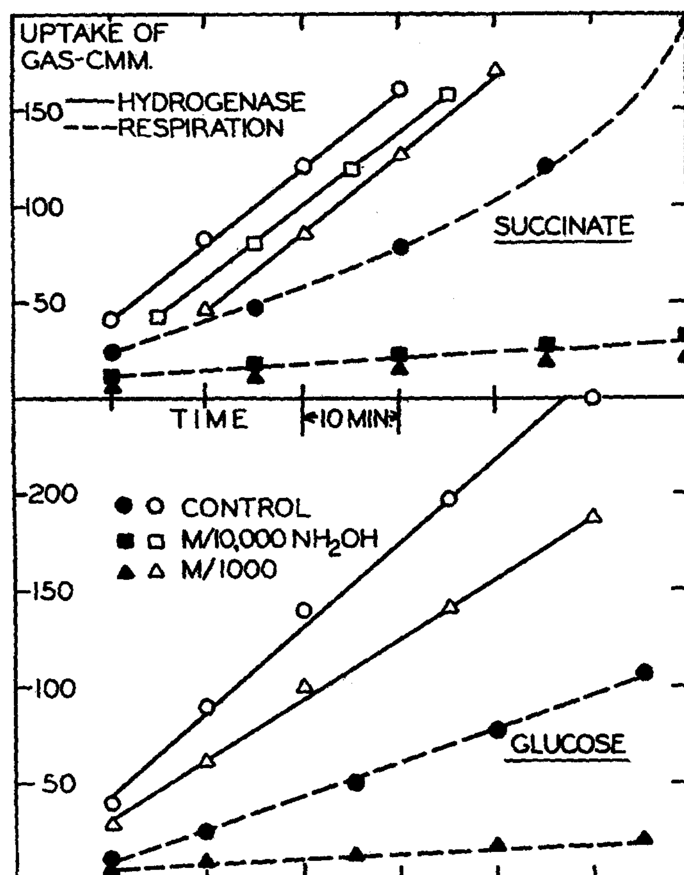

FIG. 2. Inhibition by hydroxylamine of respiration and hydrogenase in *Azotobacter vinelandii.* Hydrogenase measured in 96 per cent H_2, 4 per cent O_2; respiration, in air. Upper; succinate as respiration substrate; no effect of NH_2OH on hydrogenase. Lower: glucose as substrate; $Q_K(N)$ in absence of NH_2OH, 2260; in presence, 1740. Legend applies to both sections of figure.

succinate may be almost entirely suppressed with M/1000 to M/500 NH_2OH, oxidation of H_2 is not markedly reduced (0 to 25 per cent). If pyruvate is supplied the organism, the inhibition of respiration disappears, undoubtedly because the NH_2OH is detoxified by combination with the carbonyl group of the substrate.

Iodoacetate and Fluoride.—The results with sodium fluoride and iodoacetate are given in Table III. Differential inhibition of respiration and hydrogen oxidation by both is definite, but is especially outstanding with M/100 sodium

TABLE II

Effect of NH_2OH on Respiration and Hydrogenase in Azotobacter vinelandii

Experiment No.	Concentration of inhibitor	Respiration		Hydrogenase	
		Q_{O_2}(N)	Inhibition	Q_K(N)	Inhibition
5	None	4000	—	3520	—
	M/10,000	370	90	3680	−4
	M/5000	350	90	3460	0
	M/1000	350	90	3580	0
	M/500	75	98	3380	4
	M/100	180	95	500	86
6	None	{1560, 2150}	—	2590	—
	M/1000	0	100	1940	25

Respiration: M/100 succinate in air in Experiment 5; M/150 succinate in 96 per cent He, 4 per cent O_2 in Experiment 6.

Hydrogenase: no added substrate, 96 per cent H_2, 4 per cent O_2 in both experiments.

TABLE III

Action of Iodoacetate and Fluoride on Respiration and Hydrogenase in Azotobacter vinelandii

Inhibitor	Concentration	Respiration		Hydrogenase	
		Q_{O_2}(N)	Inhibition	Q_K(N)	Inhibition
None	—	9560	—	4340	—
Iodoacetate	M/1000	5860	39	4300	0
	M/100	760	92	4300	0
Fluoride	M/25	9900	−4	4710	−8
	M/12.5	6400	34	4410	0

Respiration: M/150 glucose in air.

Hydrogenase: no added substrate in 96 per cent H_2, 4 per cent O_2.

iodoacetate. The absence of inhibition with both fluoride and iodoacetate suggests that the oxidation of H_2 does not involve a phosphorylation step.

Hydroxylamine As a Specific Inhibitor for Biological Nitrogen Fixation

The experiments with NH_2OH explain some curious results reported a few years ago by Kubo (1937) who found that, as the concentration of this inhibitor was varied from M/10,000 to M/250, oxygen uptake by suspensions of

Azotobacter chroococcum on mannitol was materially decreased in air but not in a mixture of 80 per cent H_2 and 20 per cent O_2. He interpreted this finding as evidence for a *specific* inhibition of nitrogen fixation by NH_2OH which might be regarded as indirect support for the view that hydroxylamine is the key intermediate in the chemical mechanism. Burk was unable to confirm this specific inhibition and has questioned the validity of the hydroxylamine hypothesis for nitrogen fixation by *Azotobacter* (Burk, 1937; Burk and Burris, 1941; Burk and Horner, 1935).

Kubo's results now admit a more logical but less spectacular explanation; *viz.*, that he unknowingly was dealing with the differential effect of NH_2OH on respiration and hydrogen oxidation by *Azotobacter*. Unfortunately, he did not include an exact control (flask to which no inhibitor was added), but it appears probable that his experiments are readily and simply explained as follows. In the air series at the lowest concentration of NH_2OH (M/10,000) there remained considerable residual respiration which was effectively eliminated on further additions of the inhibitor. In the hydrogen-oxygen series, however, the gas uptake in the presence of M/10,000 NH_2OH represents the total for two competing reactions: oxidation of mannitol and of H_2. As the respiration in this series is inhibited by additional NH_2OH, the decrease in O_2 consumed is replaced by a more or less equivalent quantity of H_2 and O_2 which disappears in the *Knallgas* reaction.

If the foregoing explanation is correct, the same type of data should be obtained with any differential inhibitor. As is illustrated in Fig. 3, this is actually the case. In these experiments we have used the proper control with the following results: respiration of mannitol is almost completely inhibited by M/1000 NH_2OH or M/500 sodium azide, a compound which hitherto, at least, has not been suggested as an intermediate in biological nitrogen fixation. The reduction in gas uptake in the H_2-O_2 mixture is much smaller than in air since only the part due to oxidation of mannitol has been eliminated, the oxidation of H_2 probably remaining essentially the same. This leads to a spurious "specific" inhibition of biological nitrogen fixation; hence this particular support for the hydroxylamine hypothesis will have to be discarded.

Detection of Hydrogenase in Organisms with High Endogenous Respiration

The differential inhibitors allow a modification of our previously described method for detection of hydrogenase (Wilson, Lee, and Wilson, 1942) which should be useful with organisms in which the endogenous respiration is high. Preliminary trial of the modified method was made with *Azotobacter* cells in order to test a species known to contain the enzyme. A gas mixture of 80 per cent H_2 and 20 per cent O_2 was compared with air, and no special precautions were taken to remove substrate by repeated washings.

In agreement with our previous observations hydrogenase activity is appre-

ciably decreased in the presence of 20 per cent O_2 unless a high concentration of cells is used (Table IV). Although the cells were not repeatedly washed,

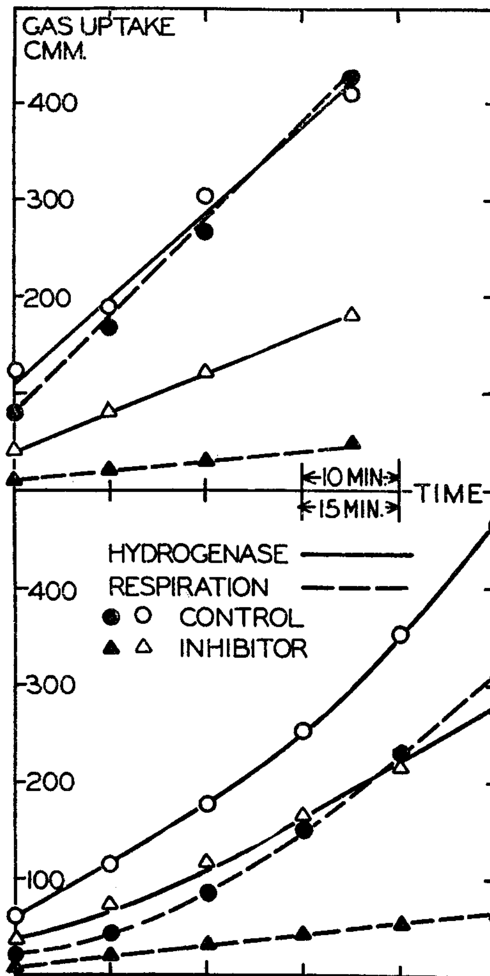

FIG. 3. Action of hydroxylamine and sodium azide on oxidation of mannitol and hydrogen by *Azotobacter vinelandii*. Respiration on M/100 mannitol in air; hydrogenase on M/100 mannitol in 80 per cent H_2—20 per cent O_2. Upper: M/1000 NH_2OH as inhibitor; Lower: M/500 NaN_3 as inhibitor. Legend applies to both sections.

the respiration was small in comparison with the gas uptake due to oxidation of H_2. Even under these conditions the differential effect of the inhibition by NH_2OH is evident.

The usefulness of the modification is best illustrated by the data of Experi-

ment 10 in which the medium used to wash the cells from the agar was employed as the suspending fluid. A respirable substrate is thus available, and as already noted (Wilson *et al.*, 1942) in this case an active hydrogen oxidation

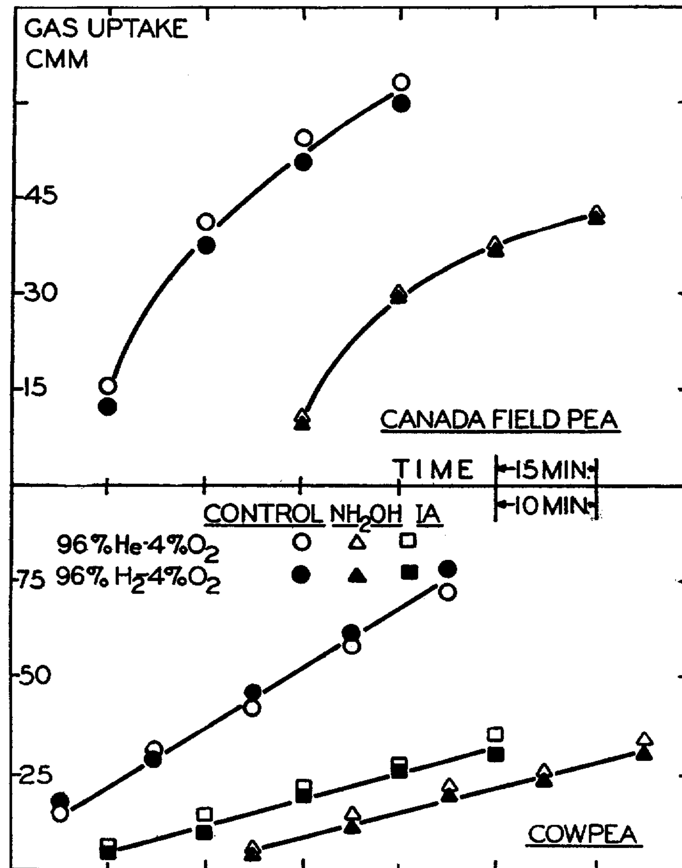

FIG. 4. Test for hydrogenase in suspensions from nodules of pea and cowpea using differential inhibitors. Hydrogenase measured in 96 per cent H_2, 4 per cent O_2; respiration control in 96 per cent He, 4 per cent O_2, no added substrate. Pea, 0.3 mg. N per flask; cowpea, 0.8 mg. N per flask. Legend applies to both sections; IA—sodium iodoacetate.

occurs even with a low concentration of cells and a pO_2 of 0.2 atm. The hydrogenase activity, however, is partially obscured by the accompanying high rate of respiration. If M/1000 NH_2OH is added, the Q_K is hardly affected, but the Q_{O_2} is reduced appreciably so that the presence of a hydrogenase is unmistakably demonstrated. Although the modified method allows the *Azoto-*

bacter suspension to be made directly from the growth on agar and the use of air for the respiration control, it would not be particularly advantageous with this organism. It should be of definite value, however, with suspensions from nodules since the bacteria in these have a gum coating which serves as substrate.

Data in Fig. 4 illustrate the use of the modified method. Suspensions of root nodule bacteria from pea and cowpea nodules, prepared by the method of Thorne and Burris (1940) were tested for hydrogenase. The relatively high

TABLE IV

Effect of NH_2OH on Respiration and Hydrogenase in Washed and Unwashed Cells of Azotobacter

Experiment No.	Species	Treatment	Cells	M/1000 NH_2OH	$Q_{O_2}(N)$	$Q_K(N)$
			mg. N/flask			
8	*vinelandii*	Unwashed	0.32	−	160	4930
				+	134	4770
					113	4550
	chroococcum	Unwashed	0.19	−	276	4650
						5260
				+	182	4260
						4460
9	*vinelandii*	Washed	0.08	−	76	710
				+	18	710
		Unwashed		−	360	1040
				+	43	1325
10	*vinelandii*	Unwashed + supernatant	0.09	−	2000	3000
				+	1240	2890
		Washed		−	133	666
				+	66	555

Respiration in air, hydrogenase in 80 per cent H_2, 20 per cent O_2.

Unwashed: growth on agar suspended in Burk's salt solution and centrifuged; supernatant discarded, and dilution made with fresh Burk's salt solution.

Washed: cells washed two additional times in centrifuge tube before dilution.

endogenous respiration is largely eliminated by NH_2OH or iodoacetate. The test for hydrogenase is negative since both the total gas uptake and the effect of the inhibitors is the same in the H_2-O_2 as in the He-O_2 mixture. The results with the suspension from pea do not entirely agree with previous ones obtained with the methylene blue technique (Phelps and Wilson, 1941) since in several instances positive results have been observed with this organism. Previous tests on bacteria taken from the soybean nodule, however, have been negative even with the methylene blue technique. This problem is being further investigated using an improved methylene blue method together with the modified gasometric method.

SUMMARY

The inhibitors usually associated with the activity of the cytochrome oxidase system—cyanide and carbon monoxide—are also effective in reducing the oxidation of H_2 by intact cells of *Azotobacter vinelandii*. The hydrogenase system is more sensitive to CO than is the respiratory system.

Oxidation of a carbon source and of hydrogen by *Azotobacter* cells is inhibited in a quantitatively different manner by the following compounds: sodium azide, hydroxylamine, sodium iodoacetate, and sodium fluoride. In every case, a concentration range which is definitely inhibitory for respiration has little or no effect on the hydrogenase activity.

The differential inhibition by hydroxylamine explains certain observations in the literature which have been erroneously interpreted as demonstrating a specific inhibition by NH_2OH of biological nitrogen fixation. This supposed demonstration has been offered as support for the hypothesis that NH_2OH is an intermediate in the fixation reaction.

The differential inhibitors can be used for detection of hydrogenase in cultures possessing a high endogenous respiration. The method is illustrated by an experiment with root nodule bacteria from pea and cowpea nodules. No hydrogenase was found in either.

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