MECHANISM OF BIOLOGICAL NITROGEN FIXATION

IX. PROPERTIES OF HYDROGENASE IN AZOTOBACTER*

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Recently Phelps and Wilson (1) reported the occurrence of hydrogenase, the enzyme which activates molecular hydrogen, in nitrogen-fixing bacteria. Because H_2 specifically inhibits nitrogen fixation by red clover plants inoculated with *Rhizobium trifolii* as well as by *Azotobacter* (2, 3), possession of hydrogenase by these organisms may be more than merely fortuitous. The possibility must be considered that hydrogenase is associated with nitrogenase, the enzyme concerned in the first step of the fixation reaction. Evidence which supports the view of a relationship was their observation that cultures of the root nodule bacteria taken directly from nodules of the pea plant fixing nitrogen contained hydrogenase but not those grown on laboratory media (on which they are unable to fix N_2). This significant finding, however, could not always be confirmed with cultures from the nodules, apparently because the factors which affect hydrogenase activity in nitrogen-fixing bacteria were not known. In the preliminary investigations we followed the technique described by Stephenson and her associates (4) for studies of hydrogenase in *Escherichia coli* and related species, but this may not be satisfactory, since the metabolism of the nitrogen-fixing bacteria is quite different from that of the colon group. We have therefore determined the chief properties of hydrogenase as it occurs in *Azotobacter* in order to develop a method which will insure consistent results in the further study of its possible relationship to nitrogen fixation.

Methods

All experiments were made with a culture of *Azotobacter vinelandii* which under optimum conditions fixes 15 to 20 mg. of N per 100 ml. in 24 hours (5). Resting cell suspensions in Allison's solution were prepared by the method of Wilson (6) from 36 hour cultures grown in Roux bottles on Burk's nitrogen-free medium plus 2 per cent agar. Gas exchanges were measured by the usual Warburg micro respirometer technique. Unless otherwise stated, 1 ml. of cell suspension and 2 ml. of phosphate buffer (pH 7.5) were placed in each flask; the temperature of the bath was 34–35°.

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Concentration of Cells

When H_2 from which all trace of O_2 had been removed was supplied to Warburg flasks containing *Azotobacter* suspensions, little or no uptake of gas occurred unless suitable acceptors (fumarate, nitrate, methylene blue) were added. When methylene blue was used, uptake of H_2 quantitatively equal to that necessary for complete reduction of dye was noted.[1] Following the suggestion of Phelps and Wilson (1) that molecular oxygen may serve as a hydrogen acceptor in this system, cell suspensions were supplied with a gas mixture of 80 per cent H_2 and 20 per cent O_2. For controls the hydrogen was replaced with helium, argon, or nitrogen (air). In a typical experiment the $Q_K(N)$ in the H_2-O_2 system was 3500 as compared with a

TABLE I

Effect of Cell Wash Solution and Glucose on $Q_K(N)$ and $Q_{O_2}(N)$

$Q_K(N)$ is based on the uptake of gas in an atmosphere of pO_2 0.2 and pH_2 0.8 atmosphere; $Q_{O_2}(N)$ on the uptake of gas in an atmosphere of pO_2 0.2 and pHe 0.8 atmosphere (KOH in center well).

Experiment No.	Concentration of cells	Addition	$Q_K(N)$	$Q_{O_2}(N)$
	mg. N per ml.			
1	0.56	None	2500	118
		Wash*	2150	1270
	0.056	None	430	100
		Wash	3550	1250
2	0.48	None	1870	120
		Glucose†	2080	350
	0.16	None	1000	100
		Glucose	2160	450

* Wash, 1 ml. of Allison's solution used for the first washing of the cells.

† Glucose, 0.001 M.

$Q_{O_2}(N)$ of 80 in the argon control and 100 in the air control.[2] These data furnish strong evidence that H_2 as well as O_2 is being consumed by the cells.

In the foregoing experiment a very heavy suspension of bacteria was used; so that the actual uptake of gas in the H_2-O_2 atmosphere was about 1000 c.mm. per hour. Such a large uptake cannot be accurately controlled because of manipulative and other technical difficulties; *e.g.*, diffusion effects. Dilution of the suspension caused a sharp drop in the $Q_K(N)$

[1] The high "endogenous" uptake of gas reported by Phelps and Wilson (1) likely arose from impurities of O_2 remaining in the H_2 used.

[2] $Q_K(N)$ = c.mm. of total gas uptake per hour per mg. of N in the cells; K refers to the fact that this oxidation of H_2 is frequently called the *Knallgas* reaction. $Q_{O_2}(N)$ = c.mm. of O_2 uptake per hour per mg. of N in the cells.

value. For example, in the experiment cited in Table I, a concentrated suspension of *Azotobacter* (0.56 mg. of N per ml.) possesses a Q_K(N) value of 2500; when the cells were diluted 1:10, it decreased to 430. This effect of dilution, which is frequently encountered in enzyme studies, usually signifies that some necessary soluble component of the system has been more or less completely removed by the washing procedure. Addition of the Allison's solution used to wash the cells in preparing the suspension completely restored the hydrogenase activity.

Further investigation showed that the stimulating factor in the cell wash solution was heat-stable and apparently could be replaced by a suitable substrate such as glucose or sucrose (Table I). Although the addition of either cell wash solution or substrate overcomes the technical difficulty of decrease (or even disappearance) of H_2 uptake with decrease in concentration of the cells, the method has the disadvantage that it causes the Q_{O_2} to become comparable in magnitude with the Q_K. For many purposes this is undesirable. A number of other soluble factors including riboflavin and cozymase which might have been lost in the washing were tested for ability to restore the hydrogenase activity in dilute suspensions, but none was effective. It is evident from these experiments that the concentration of washed cells used is an important factor in determining the activity of hydrogenase in cells of nitrogen-fixing bacteria and that this must be carefully controlled in a study of the characteristics of the enzyme.

pO_2 *Function*

A possible explanation of the effect of cell concentration was furnished by investigations which determined the influence of the pO_2 on the system. In these experiments, the pH_2 was maintained at 0.4 atmosphere, the remaining gas being He or N_2. As is evident from the data in Fig. 1, the optimum pO_2 varied with the concentration of cell suspension: the heavier the suspension, the higher the optimum. With heavy suspensions a pO_2 such as was used in the preceding experiments is satisfactory, but with more dilute ones such a pO_2 is much too high. These results suggest that a low pO_2 is necessary with low cell concentration; otherwise the enzyme system is inactivated by oxidation. With a high pO_2 substrate must be present to keep the enzyme reduced; this can be accomplished either by employing a heavy cell suspension or adding substrate (wash water or carbohydrate). Inhibition of hydrogenase by oxygen has been noted in numerous bacteria as well as algae (7–10).

Since it is not feasible to use very heavy suspensions because of too rapid uptake of gas, in subsequent work the cell suspension was standardized by means of turbidity measurements with a Coleman photoelectric colorimeter, so that it contained approximately 0.1 mg. of N per ml. Each

Warburg flask received 1 ml. of such a suspension and was supplied with a gas mixture in which the pO_2 was 0.025 to 0.05 atmosphere. The data obtained with two typical suspensions show that under these conditions the maximum Q_K is likely obtained (Table II).

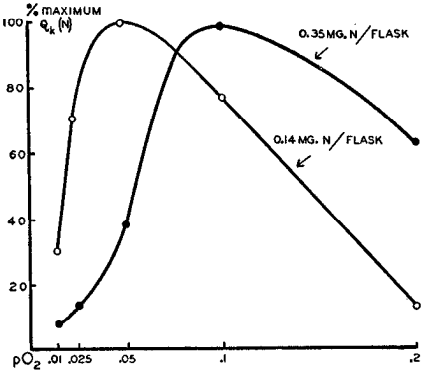

FIG. 1. Dependence on the pO_2 of hydrogenase activity in different concentrations of *Azotobacter* cells. pH_2 0.40 atmosphere, N_2 as diluent, pH 7.5, 35°.

TABLE II

Q_K (N) with Different pO_2 in Gas Mixture

Each flask contained 0.115 mg. of N in both experiments; pH_2 = 0.4 atmosphere, helium to 1.0 atmosphere.

Experiment No.	pO_2 in atmosphere				
	0.01	0.025	0.05	0.10	0.20
1	1000	2460	2085	900	363
2	1435	1610	1270	557	191

pH_2 Function

Studies on the pH_2 function summarized in Fig. 2 indicate that this is a typical activity-substrate curve. Differences in the rate of uptake of gas cannot be detected in the pH_2 range from 0.1 atmosphere to 0.95 atmosphere; below the lower limit the activity drops sharply. Because the substrate is in the gas phase, calculation of a Michaelis constant from these data may be subject to error, since diffusion of gas to the enzyme is probably a factor. Support for this view is furnished by the data of Fig. 3. With a dilute suspension of cells a K_m value of 0.025 atmosphere is indicated; with a heavier suspension, one of 0.04 atmosphere. For practical purposes, however, it appears that if a pH_2 of at least 0.4 atmosphere is used the enzyme will be saturated with substrate.

pH and Temperature Functions

The influence of pH on the system (Fig. 4) was obtained with a gas mixture containing 0.025 to 0.05 atmosphere of O_2 and the remainder H_2. The data show that the optimum pH is approximately 7.3 to 7.8. On the

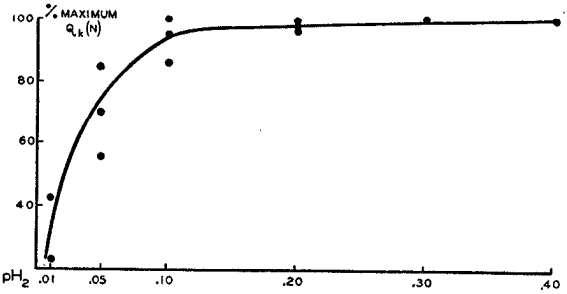

FIG. 2. Activity of hydrogenase in *Azotobacter* as a function of the pH_2. pO_2 0.025 to 0.05 atmosphere, pH 7.5, 35°.

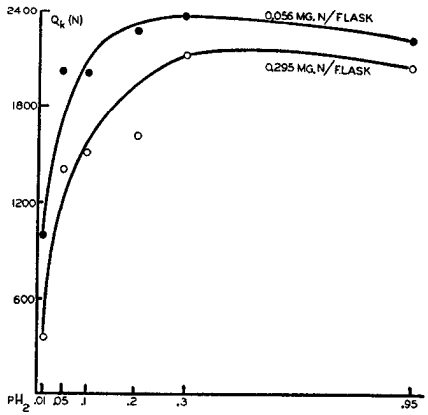

FIG. 3

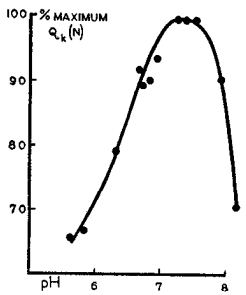

FIG. 4

FIG. 3. Change of the pH_2 function with cell concentration. pO_2 0.05 atmosphere He as diluent, pH 7.5, 35°.

FIG. 4. Response to pH of hydrogenase in *Azotobacter*. pO_2 0.025 atmosphere, pH_2 0.40 atmosphere, 35°.

alkaline side of this region the activity decreases much more rapidly than on the acid. Although the optimum pH region coincides with that for nitrogenase, the function as a whole is quite different. At pH 6.0 the activity of *nitrogenase* decreases precipitately to zero (11), whereas that for *hydrogenase* has decreased only about 30 per cent. In this respect the

pH function resembles more closely those for assimilation of different forms of combined nitrogen by *Azotobacter* (11).

The response of the hydrogenase system to temperature is illustrated in Fig. 5. The data are from two separate experiments, but the agreement is so close that it is unnecessary to adjust the observed values before they are combined. The optimum temperature is about 40°, and the μ value (6) based on the fourteen observations from 21–40° is 11,200 ± 670 calories. The corresponding constants for nitrogen fixation are 33° and 19,300 calories and for assimilation of NH_3-N, 37° and 19,300 calories. It should be noted, however, that the μ values for assimilation of free and combined

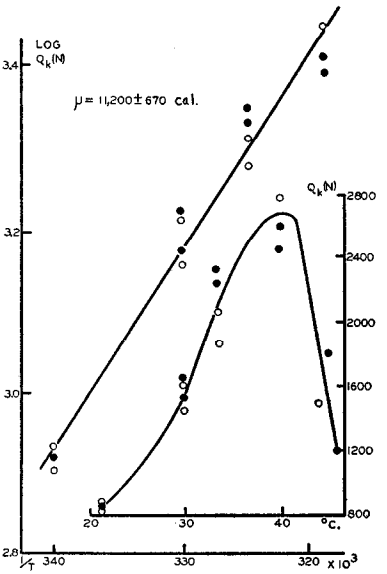

FIG. 5. The temperature characteristics of hydrogenase in *Azotobacter*

nitrogen (which are also identical with that for respiration by *Azotobacter*) were obtained with growing cultures and not with a "resting" suspension. Burk (11), who reports these values, is of the opinion that they are associated with activation of the carbohydrate component of growth rather than with the nitrogen assimilation process.

SUMMARY

The chief properties of *hydrogenase* in *Azotobacter* cells have been determined with molecular oxygen as the hydrogen acceptor. Both the pO_2 and pH_2 functions vary with the concentration of cells. The optimum pH is about 7.5 and the optimum temperature, 40°. Consideration of these

properties has led to the following method for study of hydrogenase in *Azotobacter* (and probably other nitrogen-fixing organisms). In a Warburg flask 1 ml. of a cell suspension containing 0.1 mg. of N per ml. is added to a phosphate buffer of pH 7.3 to 7.5. An atmosphere containing 0.025 to 0.05 atmosphere of O_2 and at least 0.4 atmosphere of H_2 is supplied; the temperature of the bath is kept at 34–35°. Under these conditions consistent and reproducible results are readily obtained.

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