5% to 16% of the p-nitrobenzenesulfonamide is excreted in a state of oxidation above the amine.[8]

*Conclusions.* p-Nitrobenzoic and m-nitrobenzoic acids are readily reduced to amino-compounds by tissue suspensions. o-Nitrobenzoic acid, in contrast, is reduced to only a small extent by tissue suspensions.

When p-nitrobenzoic acid or m-nitrobenzoic acid is administered to rats only a small fraction of the dose is excreted in the urine in the reduced form, the remainder is present in a state of oxidation above the amine. The reduced fraction is conjugated to a high degree. o-Nitrobenzoic acid is also reduced by rats; an appreciable amount of free amine is recoverable from the urine.

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## Occurrence of Hydrogenase in Nitrogen-Fixing Organisms.*

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Several years ago this laboratory[1] demonstrated that molecular $H_2$ specifically inhibits the assimilation of atmospheric $N_2$ by red clover plants inoculated with *Rhizobium trifolii.* In an effort to determine the mechanism of this unusual type of inhibition, cultures of the bacteria were tested for *hydrogenase,* the enzyme which Stephenson and associates[2] found in several species of heterotrophic organisms catalyzing the reversible reaction: $H_2 \rightleftarrows 2H$. Attempts to find the enzyme in cultures of *Rh. trifolii* failed. Recently Wyss and Wilson[3] showed that $H_2$ likewise inhibits nitrogen fixation by the free-living *Azotobacter.* Accordingly, species of this organism were tested for *hydrogenase,* and in contrast with the earlier results using the root nodule bacteria the azotobacter species were found to possess an active enzyme. This report presents the evidence proving the existence of the enzyme; a future paper will discuss its properties and possible significance for the mechanism of biological nitrogen fixation.

* This work was aided by a grant from the Rockefeller Foundation.

1 Wilson, P. W., *Biochemistry of Symbiotic Nitrogen Fixation,* Madison, Wis., 1940.

2 Stephenson, M. S., *Bacterial Metabolism,* London, 1939.

3 Wyss, O., and Wilson, P. W., *Nat. Acad. Sci., Proc.,* 1941, **27**, 162.

*Methods.* A modification of the Thunberg technic described by Tam and Wilson[4] in which the rate of methylene blue reduction is followed with an Evelyn photometer was used. Changes in the procedure included: solution volume increased to 10 ml; methylene blue concentration decreased to $10^{-5}$ M; cell concentration reduced so that decolorization time ranged from 30-60 minutes; 620 mμ filter used instead of 540. The tubes were twice evacuated and 0.95 atmosphere replaced with known gas mixtures bubbled through alkaline pyrogallol. All tests were made at 30°C and at pH 6.5. Azotobacter cells were grown on Burk's medium plus 2% agar; cultures of rhizobia were grown on Allison's salts plus 1% sucrose and biotin concentrate.

If the log of the percent oxidized methylene blue is plotted against time, an initial short period of lag is frequently observed, followed by a much longer period during which reduction is linear. The specific rate constant, $k$, is equal to 2.303 times the slope of the straight line. This rate constant is a much better measure of the true velocity of reduction than is the usual "time of reduction".

*Methylene Blue Reduction Studies.* Initial proof for the existence of the enzyme was furnished by the data summarized in Table I. Each tube contained only cells, buffer and the indicated atmosphere.

The values for the tubes in the vacuum represent the endogenous rate of reduction, *i. e.*, reduction in the absence of the specific substrate, $H_2$, and probably arises from dehydrogenase activity. The observed values of $k$ show that when air is replaced with He or A, reduction of methylene blue proceeds at about the same rate as the endogenous (vacuum), whereas replacement with $H_2$ causes a marked acceleration in the rate of reduction. Boiling the cells eliminated the reduction.

*Hydrogen Uptake Experiments.* Preliminary investigations were made of the activity of the enzyme by means of gas uptake experiments in the Warburg apparatus at 34°C. Side-arm flasks of 15 ml volume with provision for aeration were used; approximately one

TABLE I.

| Atmosphere in Thunberg tube | $k$ value for methylene blue reduction | | |
|---|---|---|---|
| | *Az. agile* | *Az. vinelandii* | |
| Vacuum | .0132 | .0174 | .033 |
| Helium | .0160 | .0067 | .037 |
| Argon | .0094 | — | — |
| Hydrogen | .0824 | .1196 | .212 |
| Hydrogen (boiled cells) | .0006 | .0008 | — |

[4] Tam, R. K., and Wilson, P. W., *J. Bact.*, 1941, **41**, 529.

TABLE II.

| Acceptor | $Q_{H_2}$ (N) Before addition | $Q_{H_2}$ (N) After addition |
|---|---|---|
| M.B. | 511 | 733 |
| M.B. | 503 | 652 |
| Fumarate | 578 | 822 |
| Fumarate | 413 | 800 |
| $Ca(NO_3)_2$ | 503 | 606 |
| $Ca(NO_3)_2$ | 450 | 1050 |
| Pyruvate | 750 | 750 |
| Butyraldehyde | 559 | 125 |
| Glucose | 600 | 377 |

liter of gas of the desired composition was led through each flask to displace the air. The studies showed that even without addition of specific acceptors, uptake of hydrogen was readily demonstrable, probably because of endogenous acceptors in the cells. In order to prove that a given compound could serve as an acceptor, the $Q_{H_2}(N)$ was determined first on a suspension, then on the suspension plus the compound which had been added from the side-arm. Increase in the $Q_{H_2}(N)$ following addition was taken as evidence that the compound would unite with $H_2$ in the presence of the enzyme. Typical results given in Table II show that methylene blue, fumarate, and nitrate serve as hydrogen acceptors but not pyruvate, butyraldehyde, or glucose. Each value for methylene blue, fumarate, and nitrate is from a separate experiment. Endogenous uptake of gas in a helium atmosphere was zero.

Some evidence was secured that $O_2$ might act as an acceptor for $H_2$ in experiments with resting cells of *Az. vinelandii* on glucose. The $Q_{O_2}(N)$ under an atmosphere of 80% He-20% $O_2$ was compared with that under an atmosphere of 80% $H_2$-20% $O_2$; if the cells assimilated $H_2$, the rate of uptake of gas in the $H_2$-$O_2$ atmosphere should be greater than in the He-$O_2$. In one experiment the $Q_{O_2}(N)$ for cells in the presence of He-$O_2$ was 2590 whereas in the presence of $H_2$-$O_2$ a value of 4040 was observed. Additional positive experiments, while not so striking, showed definitely greater rates in the $H_2$-$O_2$ atmosphere, but in others no significant differences were noted.

*Miscellaneous Observations.* The effect of cyanide and urethane on the activity of the enzyme in *Az. vinelandii* is illustrated in Table III.

These responses are typical of dehydrogenases in general and of hydrogenases reported for other bacteria.

The effect of environment on the occurrence of the enzyme was

TABLE III.

| KCN | $k$ value | Urethane | $k$ value |
|---|---|---|---|
| Endogenous | .0053 | Endogenous | .0465 |
| Hydrogen | .0639 | Hydrogen | .1610 |
| " + M/200 KCN | .0641 | " + M/80 | .1207 |
| " + M/100 | .0685 | " + M/8 | .0940 |
| " + M/75 | .0720 | | |
| " + M/50 | .0694 | | |
| " + M/20 | .0083 | | |

determined by cultivating *Az. vinelandii* on a nitrogen-free agar medium and on the same medium plus 200 mg/100 ml of $NH_4NO_3$. Flasks of both series were filled with the following atmospheres by the method of Wyss and Wilson[3]: (1) air; (2) $pO_2$, 0.2, $pN_2$, 0.2, $pH_2$, 0.6 atm.; (3) $pO_2$, 0.2, $pN_2$, 0.3, $pH_2$, 0.5 atm. Values of $k$ for the reduction of methylene blue in an atmosphere of $H_2$ were then determined for the six suspensions prepared from these treatments after adjusting each to a standard reduction time. Results from this preliminary study indicated that the enzyme is produced by azotobacter cells grown under all the conditions used.

A reinvestigation of the occurrence of *hydrogenase* in the rhizobia resulted in a quite unexpected observation. In agreement with our earlier findings, cultures of *Rh. leguminosarum* 311 grown on laboratory media do not possess the enzyme but suspensions taken by the method of Thorne and Burris[5] directly from the nodules of peas inoculated with this strain of rhizobia actively reduce methylene blue in the presence of $H_2$. Preliminary trials with the soybean organism, however, indicate that neither laboratory nor nodular cultures possess the enzyme.

*Summary.* The existence of *hydrogenase,* the enzyme activating molecular $H_2$, has been demonstrated in cultures of *Azotobacter* by a methylene blue reduction method as well as by measurements of $H_2$ uptake in the Warburg microrespirometer. The enzyme is found in cells grown in the presence or absence of $H_2$ and using either free or combined nitrogen. Cells of the pea organism taken directly from the nodule possess the enzyme but those grown on the usual laboratory media do not.

[5] Thorne, D. W., and Burris, R. H., *J. Bact.*, 1940, **39**, 187.