THE MECHANISM OF BIOLOGICAL NITROGEN FIXATION[1]

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In late 1940 two summary reports concerned with biological nitrogen fixation were completed, the monograph, *Biochemistry of Symbiotic Nitrogen Fixation*, by Wilson and the review by Burk and Burris which appeared the following year in *Annual Review of Biochemistry*. The judgment of these three authors at that time regarding the chemical mechanism of the process is expressed in these quotations from the review,

"For many workers it has been psychologically irresistible to conclude upon finding some extracellullar nitrogenous product that it was involved in either the initial or later anabolic rather than catabolic stages. Although a considerable number of isolated and hypothetical compounds have been so regarded during the past fifty years, the main controversy during the past decade has been confined to the two rival intermediates, ammonia and hydroxylamine (or oxime). The present weight of evidence favors the latter, but by no means offers unequivocal support.

". . .the occurrence of any ammonia, observed or otherwise, as an essential intermediate in nitrogen fixation by *Azotobacter*, admittedly still possible, yet awaits sufficiently critical experiments in support."

Since these words were written, we believe that sufficient critical experiments have been completed and that their results, together with relevant data obtained in the previous decade, warrant a reversal of the opinion expressed in the last lines. Moreover, present established facts may even justify an attempt to draw a tentative blueprint of possible mechanisms, especially since there have been no frankly theoretical papers in the field since those of Burk (1934, 1937). Even though such schemes represent little more than an enlightened surmise, they should be helpful in evaluating the advances that have been made, in noting the gaps in our information, and in suggesting possible methods for filling these. Before discussing these recent studies in detail, we should, however, first consider briefly the developments that led to them.

Until about 1930 the little appreciated difficulty of insuring purity of Azotobacter cultures (Winogradsky, 1937; Burk and Burris, 1941 p. 604) was so neglected that most early papers possess only historical interest. The inherent complication of the symbiotic fixation requiring as it does two organisms, bacteria and plants, discouraged attempts to investigate the mechanism of this system. The modern studies may be said to date from the paper of Meyerhof and Burk (1928), which described a method for using the micro-respirometer to investigate the mechanism of nitrogen fixation by Azotobacter. Important as was the introduction of this technique whose usefulness already had been demonstrated in enzyme studies, their major contribution was applying the

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point of view of physical chemistry to the problem. Investigations of the mechanism by precise physical-chemical methods led to a much-needed more critical attitude toward what constituted admissible evidence for support or rejection of a particular hypothesis. Burk (1936, 1937) proposed seven criteria for examination of claims, and whether one agrees with him as to either their necessity or sufficiency, they remind the investigator that standards here as in other branches of science are essential.

While Burk and his associates continued the physical-chemical studies with Azotobacter, Wilson and his collaborators at the University of Wisconsin began analogous investigations using the symbiotic system of inoculated red clover plants. These researches by the two American groups eventually provided much necessary detailed information about the characteristics of the enzyme system responsible for fixation. By their very nature, however, they could furnish only suggestions about the chemical intermediates. Experiments on this question were made by Virtanen (1938) and his coworkers in Finland with the symbiotic systems and by Winogradsky (1930, 1935, 1938, 1939) in France using Azotobacter. The physical-chemical combined with the organic approach first yielded definite and precise information on intermediates when Burris (1942) attacked the problem by application of isotopic techniques.

IDENTIFICATION OF THE INITIAL KEY INTERMEDIATE

I. Hydroxylamine. Although numerous compounds have been proposed for the key intermediate position in biological nitrogen fixation—representing the end-product of the initial stage of fixation and the starting point for assimilation —only two, ammonia and hydroxylamine, are backed by serious experimental attempts at verification. Hydroxylamine, first suggested by Blom (1931), has received its experimental support almost entirely from the studies of Virtanen and his associates (Virtanen, 1938, 1939; Virtanen and Laine, 1939) on the excretion phenomenon in inoculated leguminous plants. The main pillars are:

1. Aspartic acid is the sole nitrogenous compound excreted in any quantity.
2. Extremely small quantities of an oxime, tentatively identified as oximinosuccinic acid, is detected in the excretory products.
3. Oxalacetic acid is found in leguminous plants.
4. Excised nodules fix more nitrogen when oxalacetic acid is supplied.

We have already examined these claims in detail and rejected their specificity (Wilson, 1939, 1940, p. 173; Burris and Wilson, 1945). Briefly, our view is that most of the evidence could be applied equally well in support of the ammonia hypothesis. The most specific—the isolation of the oxime—suffers from the fact that the quantity found is extremely small. Not only does this place the compound in the category of many other nitrogenous products traces of which are found in cultures and substrates of nitrogen-fixing organisms, but also it interposes difficulties in the essential unequivocal identification. A recent publication by Virtanen, Linkola, Hakala, and Rautanen (1946), however, reduces the entire question to an academic status as far as its significance for the hydroxylamine-ammonia controversy is concerned. They report that, contrary

to their earlier findings, the excretion products consist not entirely of aspartic acid, β-alanine and oximinosuccinic acid but of a mixture of these and *glutamic* acid. Hence, if the experiments on excretion are to be accepted as having a necessary significance for the identity of the key intermediate, a view that Wilson and Wyss (1939) questioned, the new work includes ammonia as well as hydroxylamine.

Experiments that implicate hydroxylamine with fixation of N_2 by Azotobacter are confined largely to reports that traces of oximes have been detected in cultures. Endres and his co-workers (1934, 1935, 1938) contend that they have associated specifically the occurrence of the oxime with assimilation of N_2; but Burk (1937) questions this claim on the grounds that an oxime is also found in cultures supplied with fixed nitrogen including ammonia, and that the oxime is not used for growth by Azotobacter. Finding NH_2OH in cultures of *Aspergillus niger* supplied with ammonium salts, Steinberg (1939) concluded that it arose from oxidation of the ammonia. Therefore, occurrence of hydroxylamine or its oximes can hardly be regarded as a biological rarity and therefore necessarily associated with the specific process of fixation of N_2.

II. Ammonia. Experiments with the stable nitrogen isotope N^{15} by the authors and their collaborators have supplied an array of evidence that definitely points to ammonia as the key intermediate in nitrogen fixation by Azotobacter. Four specific types of evidence can be cited, specific in the sense that the observations depend on the demonstrated functioning of the nitrogen fixation reaction.

1. In experiments of short duration N^{15} supplied as either N_2 or NH_4^+ accumulates in the same fractions, notably glutamic and aspartic acids. Later a more general distribution in other fractions appears (Burris, 1942; Burris and Wilson, 1946). Typical results from such experiments are shown in table 1. In this table the most significant data are those in which the N^{15} concentration exceeds that of the average represented by "total hydrolysate". In an extremely short-time experiment with labeled ammonium (3 minutes) the highest level of isotopic nitrogen is found in the glutamic acid fraction—"amide" nitrogen includes ammonium nitrogen and hence absorbed $N^{15}H_4^+$. After 15 minutes a more general distribution of N^{15} is noted, but by far the highest concentration of labeled nitrogen is still maintained in the glutamic acid fraction. Compare now these distributions when NH_4^+ is supplied with that observed in Azotobacter fixing N^{15}-enriched N_2 for 90 minutes when equilibrium conditions would be more closely approached. The similarities (high N^{15} levels in glutamic and aspartic acids, low levels in arginine, histidine, etc.) point unmistakably to the functioning of NH_4^+ in the fixation reaction. Additional support is provided by the fact that not only in Azotobacter but also in both plants and animals supplied N^{15} as NH_4^+ the N^{15} accumulates in glutamic acid indicative that this reaction is of general biological significance (Foster, Schoenheimer and Rittenberg, 1939; Rittenberg, Schoenheimer, and Keston, 1939; Vickery, Pucher, Schoenheimer and Rittenberg, 1940). The secondary accumulation observed in the aspartic acid fraction probably reflects the activity of the glutamic-aspartic transaminase in Azotobacter (Lichstein and Cohen, 1945). The argument that the fixation

might proceed via the NH_2OH-aspartic route and that the low concentration of this amino acid as well as its inferior N^{15} content arises from its transfer of fixed N^{15} to α-ketoglutaric acid ignores the fact that the equilibrium favors the formation of aspartate not glutamate, and that it would be impossible to pass N^{15} at a particular concentration in aspartate to build up a higher concentration in glutamate.

In an experiment such as the ones described, passing N^{15} at a particular concentration from aspartate to build up a higher concentration in glutamate would be impossible, as stated, without making the illogical assumption that during the short period when N^{15} was present the organisms suddenly shifted the ratio of aspartate to glutamate formed.

TABLE 1

Distribution of N^{15} in Amino Acids and Amino Acid Fractions

	ATOM PER CENT N^{15} EXCESS		
	15 min. NH_4^+ treatment	3 min. NH_4^+ treatment	90 min. N_2 treatment
Total hydrolysate	1.049	0.174	0.275
Humin	0.683	0.067	—
Neuberg filtrate	0.915	—	0.441
"Amide" nitrogen	0.981	0.749	0.325
Arginine	0.667	0.026	0.185
Glutamic acid	2.594	0.392	0.500
Aspartic acid	0.832	0.187	0.376
Histidine fraction	0.634	0.034	0.207
Lysine fraction	0.807	0.052	0.356
H_2O-insoluble Cu salts	0.690	0.075	0.313
H_2O-soluble MeOH-insoluble Cu salts	0.617	0.039	0.320
H_2O-soluble MeOH-soluble Cu salts	0.782	0.042	0.327
Tyrosine	0.758	—	—
Leucine	0.602	0.068	—

To illustrate, if N^{15} is supplied at 30 atom per cent excess concentration for a period during which the culture makes a tenth of its growth all nitrogenous compounds in the cell would contain 3 atom per cent excess N^{15} if the fixed nitrogen were immediately transferred directly to all of them and if all these compounds were being formed at the same rate as they had been during the period when excess N^{15} was absent. However, such is not observed; instead the concentration differs in the substances actually isolated. The high atom per cent excess N^{15} in a given compound could be explained by assuming that when the N^{15}-enriched N_2 or NH_4^+ is added, glutamic acid, for example, is suddenly produced at a more rapid rate than formerly; that is, if its rate of formation were doubled it would contain 6 rather than 3 atom per cent N^{15} excess. There is, however, no logical basis for such an assumption, since N^{15} is metabolized in the same manner as N^{14}. The high N^{15} concentration in glutamic acid can also be explained on the simple assumption that when nitrogen is fixed the product of fixation is transferred by a chain of reactions to form the nitrogenous compounds in the cell. For example, suppose that the following schematic chain mechanism of nitrogen fixation and assimilation has been in operation and has built up all the products shown:

$$N_2 \rightleftharpoons 2AN \rightleftharpoons 2BN \rightleftharpoons 2CN$$

Now if a source of N^{15}-enriched nitrogen is furnished for a short time, and if the total reaction is then stopped before equilibrium is reached, the highest level of N^{15} will be in the source supplied, for obviously no reaction could selectively effect an increase over this level. The next highest N^{15} concentration (all expressions are in terms of concentration, not absolute amount) would be in AN, for it lies closest to the original source of N^{15} and all subsequent reactions are dependent upon it. Likewise BN will have a higher N^{15} concentration than CN. *A high concentration* (atom per cent N^{15} excess) *of N^{15} indicates proximity in the reaction chain to the initial N^{15}-enriched compound supplied.* A stepwise transfer of N doubtless occurs in Azotobacter and to us presents the only logical explanation for the observed differences in the atom per cent N^{15} of isolated compounds. The high atom per cent N^{15} excess in glutamic acid noted in Azotobacter supplied N^{15}-enriched N_2 (or NH_4^+), therefore, indicates the proximity of glutamic acid to N_2 (or NH_4^+) in the nitrogenous metabolism of Azotobacter.

TABLE 2

Competition between free and combined forms of nitrogen by Azotobacter vinelandii

FIXATION IN PRESENCE OF	NON-ADAPTED CULTURES[a]	ADAPTED CULTURES[b]
	per cent	*per cent*
N_2	100	100
NH_4^+	0.0	0.7
NO_2^-	14.2	12.2
NO_3^-	20.0	1.1
Urea	—	1.2
Asparagine	73.7	48.9
Aspartate	91.4	84.2
Glutamate	96.7	89.0
Arginine	104.8	101.5

[a] Cultures previously kept on N-free medium.

[b] Cultures immediately before use transferred daily for 3 successive days on medium containing source of combined nitrogen to be tested.

Figures in table give percentage of total nitrogen in the cells that has come from N_2 in presence of combined nitrogen indicated in column 1.

Actual isotopic analyses given by Wilson, Hull and Burris (1943).

2. Ammonia is completely and immediately accepted as a source of nitrogen to the exclusion of the nitrogen fixation reaction; this occurs independently of whether the organism has been cultivated earlier on N_2, NH_4^+ or NO_3^- (Burris and Wilson, 1946; Wilson, Hull and Burris, 1943). Compounds readily converted into ammonia, as urea, are also effective in complete inhibition of fixation.

3. Compounds less readily converted into ammonia, as nitrate or nitrite, require a period of adaptation before they effectively inhibit fixation; this indicates that these compounds do not inhibit *per se* but only after the organism develops enzyme systems that convert them into an inhibitory product. Organic nitrogen compounds, which represent products somewhat removed from the assimilating system concerned with N_2 and inorganic combined sources of nitrogen, inhibit fixation only moderately.

Wilson, Hull and Burris (1943) and Burris and Wilson (1946) furnish evidence from isotopic experiments supporting points 2 and 3. Table 2 gives typical

results showing how ammonia and urea completely replace the nitrogen fixation reaction even without adaptation in contrast to all the other nitrogenous compounds tested. Figure 1 illustrates that the displacement of the fixation by the NH_4^+ assimilation is almost instantaneous. Calculations based on the recovery of N^{15} indicate that the shift from fixation of N_2 to utilization of NH_4^+ is not only rapid but total, so that ammonia completely inhibits fixation until it is exhausted (Burris and Wilson, 1946). Figure 1 also shows that a considerable period of adaptation is necessary before Azotobacter uses nitrate; its assimilation then

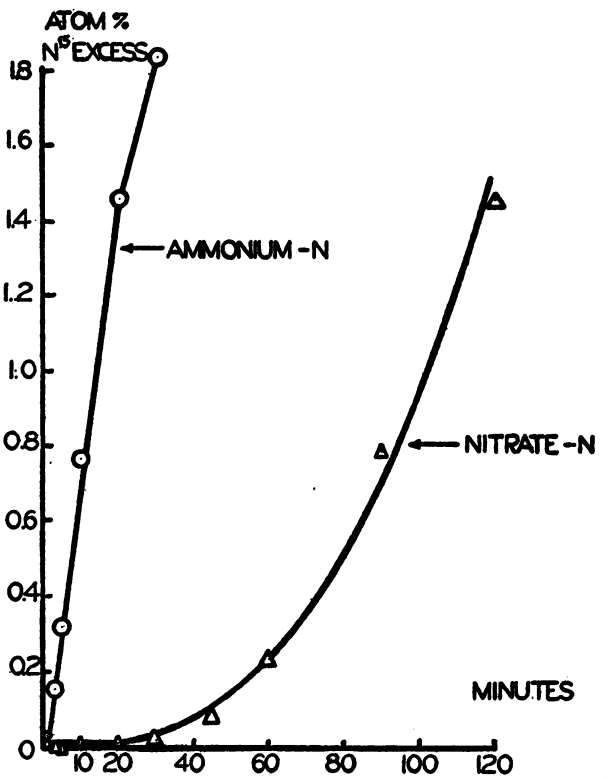

FIG. 1. Comparison of uptakes of ammonium and nitrate nitrogen by *Azotobacter vinelandii* (from Burris and Wilson, 1946)

proceeds at an increasing rate. The inhibitory product that is formed from nitrate (and presumably nitrite) after the period of adaptation is undoubtedly ammonia as was demonstrated by isotopic experiments in which Azotobacter was supplied with $N^{15}H_4NO_3$. Isotopic nitrogen ($N^{15}H_4^+$) disappeared from the medium more rapidly than did total NH_4^+ indicating that NO_3^- was being converted into NH_4^+. The residual NH_4^+ constantly decreased in its N^{15} content as can be seen in column 5 of table 3. It is also noted in this table that in the presence of both, NH_4^+ is used in preference to NO_3^-. The initial N^{15} content of the NH_4^+ was 28.90 atom per cent excess; if all the cellular nitrogen had arisen

from NH_4^+ or NO_3^-, the atom per cent excess in the cells would have been 28.90 or 0.00, respectively, whereas equal utilization of NH_4^+ and NO_3^- would give 14.45 per cent. The observed level at 8 hours (table 3, column 3), 19.62 atom per cent excess, indicates a preferential use of NH_4^+. Preferential use of NH_4^+ has also been observed with cultures previously adapted to NO_3^-.

Likewise, the limited inhibition noted with the organic compounds (table 2) probably depends on splitting off ammonia from these. Since asparagine requires only hydrolysis for liberation of ammonia, it is a fairly effective inhibitor especially with adapted cultures. Liberation of ammonia from glutamic acid, aspartic acid and arginine likely necessitates an oxidative deamination with a number of steps, so that conversion is either incomplete or at a rate too slow to compete appreciably with the fixation of nitrogen.

Although the most convincing results have been obtained with the isotopic technique, microrespirometer and macro total nitrogen experiments provide confirmatory indirect evidence. That NH_4^+ is an intermediate in the utilization of NO_3^- by Azotobacter is suggested by the observation that a lag exists in the

TABLE 3

Uptake of ammonia- and nitrate-N by Azotobacter vinelandii

HOURS AFTER INOC.	TOTAL N IN CELLS	ATOM % N^{15} EXCESS IN CELLS	NH_4^+-N IN MEDIUM	ATOM % N^{15} EXCESS IN NH_4^+	NO_3-N IN MEDIUM
	mg/100 ml		*mg/100 ml*		*mg/100 ml*
0	—	—	7.46	28.90	7.80
8	2.92	19.62	6.69	24.94	5.49
14	9.04	16.67	3.29	13.63	3.08
20	13.61	13.91	1.31	9.65	0.62
24	14.43	13.48	0.85	7.69	0.26

uptake of NO_3^-, but this lag can be eliminated if a small quantity of NH_4^+ is provided (Lind and Wilson, 1942, fig. 5). In our extensive studies (Lind and Wilson, 1941, 1942; Wilson, 1940; Wilson and Lind, 1943; Wyss and Wilson, 1941a; Wyss, Lind, Wilson and Wilson, 1941) on inhibition of the fixation of N_2 by H_2 or by small concentrations of CO, it was frequently noted that these apparently also inhibited the assimilation of some kinds of combined nitrogen when the experiments were made in the presence of N_2 (air or even helium containing only 2 per cent N_2). This was always observed with aspartic and glutamic acids, asparagine and arginine, less frequently with nitrate (nitrite) and not at all with ammonia or urea. If N_2 was removed entirely by using a $H_2 + O_2$ mixture, the inhibition disappeared. Such results demonstrate that, except for NH_3 and urea, the combined nitrogen sources had not entirely displaced fixation when N_2 was available. Inhibition of NO_3^- assimilation was not obtained in the presence of N_2 if NH_4NO_3 was used, nor was it observed in the long-time plant experiments with nitrates of calcium, potassium or sodium. These findings suggest that uptake of combined nitrogen is inhibited by H_2 or CO only if

Azotobacter or the leguminous plant had not had sufficient time to develop systems to keep available a constant supply of NH_4^+-N.

4. Azotobacter uses the combined nitrogen in surprisingly few compounds: ammonia, urea, nitrite, nitrate, aspartic and glutamic acids, asparagine, and possibly a few others (Burk and Burris, 1941; Horner and Allison, 1944; Wilson and Lind, 1943; Wilson, Hull and Burris, 1943). Of these only ammonia or compounds readily converted to ammonia are assimilated at a rate comparable to that of free N_2. In microrespirometer trials Wilson and Lind (1943) noted the following k values.[2]; for assimilation of N_2, NH_4^+ and urea, 0.30 to 0.40; NO_3^- or NO_2^-, 0.10 to 0.15 unless adapted when the values rose to 0.30 to 0.40; asparagine, about 0.10 in unadapted cultures, 0.20 to 0.25 in adapted; aspartic acid, and glutamic acid, 0.10 or less. Similar results have been observed in macro total nitrogen experiments (Lind and Wilson, 1942). Although the *ad hoc* argument might be raised that permeability difficulties restrict the rate of assimilation of the other compounds, it is significant that special pleading is unnecessary for ammonia, it behaves as a proper intermediate. It seems more likely that the rates of growth with the other sources of nitrogen reflect the ease or difficulty of conversion to the key compound of nitrogen metabolism in the Azotobacter, namely, ammonia.

Finally, a great mass of nonspecific evidence exists that is consistent with the view that ammonia is the key intermediate in biological nitrogen fixation as well as in assimilation of combined nitrogen. For example, amides, the storage form of ammonia in plants, account for a large share of the nitrogen in nodules (Wilson, 1940, p. 183); detection of ammonia liberated from detached nodules (Demolon and Dunez, 1943; Winogradsky and Winogradsky, 1936, 1941) or in the extracellular nitrogen of Azotobacter cultures is commonplace (Burk and Horner, 1936; Butkevich and Kolesnikova, 1941; Horner and Burk, 1939; Kostytschew, Ryskaltschuk and Schwezowa, 1926; Novogrudsky, 1933; Roberg, 1936; Winogradsky, 1930, 1938, 1939, 1941). But the embarrassing wealth of this seemingly essential demonstration still remains unconvincing, since no serious effort has been made to answer the argument first made by Burk and Horner in 1936 that it is of catabolic and not intermediary anabolic origin. It would add little to revive this controversy except to note that we are in accord with the view expressed by Horner and Burk (1939) that

"the extracellular ammonia observed heretofore, in our own and in all previous investigations by others, has been derived entirely from the decomposition of normal Azotobacter nitrogen upon depletion of readily available organic matter from the medium, and not, in any measurable quantity, by direct synthesis from free N_2; the ammonia observed has been liberated *after*, not *before*, a synthesis into cell nitrogen. . . ."

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[2] $$k = \frac{2.303}{t} \log \frac{\text{respiration rate after } t \text{ hours}}{\text{respiration rate at start}}.$$

A k value of 0.1 means that the rate of respiration doubles every 6.93 hours; of 0.2, every 3.46 hours.

The detailed reasons for this conclusion have been adequately discussed by Burk and Horner (1936), Horner and Burk (1939), Wilson (1940, p. 179), Burk and Burris (1941, p. 598) and others; hence they need not be repeated here. It should suffice to state that in spite of many admittedly ingenious technical approaches used by Winogradsky we have seen no paper by him or by others that has concretely met the issues first raised by Burk and Horner over 10 years ago. Until more realistic evidence is available we believe it best to avoid reliance on such shaky experimental support. Nevertheless, and without abandoning our position, if such nonspecific evidence as mere detection of a postulated intermediate in culture or plant is to be seriously considered, it is emphasized that of all postulated intermediates, NH_4^+ is most easily isolated from cultures.

Similarly, many of the other types of nonspecific evidence in favor of hydroxylamine can be matched with corresponding observations pointing to ammonia. Whether oxalacetic acid occurs in leguminous plants, and if it does, whether it implicates NH_2OH specifically is immaterial when it is realized that both α-ketoglutaric (Virtanen, Arhimo, Sundman, and Jännes, 1943) and a possible precursor, citric acid, can be found in the same plants. The alleged stimulation of fixation by excised nodules through addition of oxalacetic acid has been achieved only by Virtanen (Allison, Hoover and Minor, 1942; Virtanen, 1939; Wilson, 1940). During the past few years we have tested more than 100 cultures of excised nodules using the precise and sensitive isotope method. As is evident from typical data in table 4 that supplement data cited earlier (Burris, Eppling, Wahlin and Wilson, 1943), only occasionally have we observed fixation, and its occurrence is as likely in the culture given α-ketoglutaric acid, citric acid or no added metabolite as in that given oxalacetic acid.

ENZYME SYSTEMS ASSOCIATED WITH N_2-ASSIMILATION

In addition to providing specific information about the chemistry of nitrogen fixation, application of physical-chemical methods has furnished the first reliable details concerning the responsible enzyme systems. As will be evident in the next section, such knowledge is necessary if progress is to be made in formulating reaction mechanisms and in testing their relative validity. Burk (1934) fathered the initial attempt to describe in quantitative terms the enzyme mechanism of fixation by Azotobacter and christened the expected issue. He proposed that the entire system catalyzing the transfer of N_2 to the fixed form be called *azotase*. In addition to the enzyme *nitrogenase*, which specifically combines with N_2, azotase included several other components as calcium (strontium), molybdenum (vanadium), and hydroxyl. Later, after more detailed investigations had indicated revision, he rejected their specificity for fixation (Burk and Burris, 1941, p. 608). It appears desirable then to drop the term azotase, especially since modern enzyme chemists have discarded the concept (*cf.*, zymase) that suggested it. Nitrogenase might be retained purely as a matter of convenience. A detailed discussion of the experimental data that led to the following summary is given in the monograph by Wilson and the several reviews already cited.

1. The Michaelis constant. The most recent estimate of the apparent

Michaelis constant, the partial pressure of nitrogen (pN_2) at which the enzyme nitrogenase is half-saturated in Azotobacter, is that of Wilson, Burris and Lind

TABLE 4

Fixation of N_2 by excised nodules from leguminous plants

NODULES FROM	TREATMENT	ATOM % EXCESS N^{15} IN NODULES
Peas	6.4 g. + 200 mg. OA; 41 hrs. 25°; 1.5% N^{15} ex. atm.	0.001
Peas	Burk's soln.	0.078
Peas	Burk's soln.	0.007
	" " ; 80% O_2 in atm.	0.035
	" " + 10 mg. HDP	0.001
	" " + " ATP	0.026
Clover	Burk's soln.; 21 hrs. 22°; 32% N^{15} ex. atm.	0.724
Clover	Burk's soln.; from CO plants	0.015
	" " ; " H_2 "	0.014
	" " ; " control "	0.028
	Nodulated roots from CO plants	0.022
	" " " H_2 "	0.054
	" " " control "	0.060
Clover	H_2O; from CO plants	0.029
	" ; " H_2 "	0.050
	" ; " control "	0.031
	Nodulated roots from CO plants	0.029
	" " " H_2 "	0.082
	" " " control "	−0.001
Soybeans	Burk's soln. + 10 mg. OA; 24 hrs. 21°; 32% N^{15} ex. atm	0.189
	Burk's soln. + 10 mg. succinic acid; 24 hrs. 21°; 32% N^{15} ex. atm.	−0.002
Soybeans	Burk's soln.; 24 hrs. 25°; 30% N^{15} ex. atm.	0.007 0.002
	" " + 10 mg OA; " " " "	0.013
	" " ; nodulated roots; " " " "	0.033 0.011
Cowpeas	H_2O; 24 hrs. 25°; 32% N^{15} ex. atm.	0.013
	" + 10 mg OA; 24 hrs. 25°; 32% N^{15} ex. atm.	0.004 0.041
Cowpeas from aseptic cultures	Burk's soln.; 40 hrs. 25°; 30% N^{15} ex. atm.	0.509 1.280 0.117
	Burk's soln; nodulated roots	0.883 1.507

OA = oxalacetic acid.
CO plants = grown in presence of small quantity of CO to inhibit fixation.
H_2 plants = grown in presence of 0.6 atm H_2 to inhibit fixation.
HDP = hexose diphosphate.
ATP = adenosine triphosphate.
ex. atm. = excess of N^{15} in the atmosphere.

(1942). Four methods were used that gave values ranging from 0.01 to 0.029 atm; 0.02 ± 0.005 atm was regarded as the most probable estimate. Because

of technical difficulties (long-time experiments, equilibrium between gas and interior of nodule), determining the K_{N_2} for the symbiotic system is subject to greater error. In red clover, Wilson (1940, p. 194) secured an approximate value of 0.05 atm, *i.e.*, of the same order of magnitude as that in Azotobacter.

2. Hydrogen as a specific inhibitor. Numerous experiments, including macro total nitrogen and microrespirometer experiments, have established that molecular H_2 inhibits fixation of N_2 by the symbiotic system in red clover (Wilson, 1940, p. 197) and by Azotobacter (Wyss and Wilson, 1941a). The inhibition is specific, since uptake of combined nitrogen in the absence of N_2 is not affected. Also, it is competitive, that is, the inhibition depends on both the pH_2 and the pN_2 (Wilson, Lee and Wyss, 1941; Wyss *et al.*, 1941). As the K_{H_2} value for Azotobacter is 0.11 atm, the affinity of N_2 for nitrogenase is about 5.5 times that of H_2.

3. Carbon monoxide as an inhibitor. Carbon monoxide at extremely low partial pressures (0.0001–0.0005 atm) inhibits nitrogen fixation in red clover (Lind and Wilson, 1941) and at concentrations ten-fold greater, in Azotobacter (Lind and Wilson, 1942; Wilson and Lind, 1943). Inhibition by CO differs from that by H_2 in that uptake of combined nitrogen is likewise inhibited if a considerably higher pCO, 0.01 to 0.05 atm, is used; a quantitatively smaller inhibition is frequently observed with NO_3^- (NO_2^-) even in the ranges associated with inhibition of fixation. Thus the inhibition appears to be concerned with the reduction of nitrogen compounds rather than N_2 specifically. The inhibition is primarily non-competitive, depending only on the pCO (Ebersole, Guttentag and Wilson, 1944). Some suggestion of a quantitatively less important competitive inhibition was noted, possibly arising from the fact that CO is an isostere of N_2. Analogous results with both hydrogen and CO have been observed recently in the blue-green alga, Nostoc (Burris and Wilson, 1946a).

4. Other inhibitors. Although most of the inhibitors commonly used in enzyme research have been tested with both Azotobacter (Burk, 1934) and the symbiotic system (Wilson, 1939), none could be specifically associated with the fixation process. This means that if these inhibitors do affect the nitrogen-fixing system, the inhibition cannot be detected, since they inhibit organisms supplied with fixed nitrogen as well. Study of the nitrogen-fixing system is still restricted by the circumstance that its occurrence is growth-bound, therefore differentiating among effects on various processes is practically impossible unless a specific effect is obtained. Claims of such specificity by Kubo (1937) for hydroxylamine are probably better accounted for by the differential effect of this compound on respiration and on oxidation of H_2 by a hydrogenase (Wilson and Wilson, 1943). The recent report of Fedorov (1946) concerned with the action of narcotics on nitrogen fixation might also be regarded as implying specific inhibition.

5. Auxiliary constituents. As has been mentioned, Burk (1934) believed that the nitrogen-fixing system in Azotobacter possessed components associated with calcium, molybdenum and hydroxyl, but later rejected this idea on the grounds that further experiments had demonstrated that these ions were not uniquely required for fixation but also for growth when the organism was supplied fixed

nitrogen. Although quantitative differences were evident, he believed that these might arise from differences in strains, rates of growth, differing temperature optima and other factors. Observe that these considerations do not rule out their functioning in the nitrogen-fixing system; the restriction that we must study the reaction in growing organisms, however, makes it difficult, if not impossible, to differentiate among effects. Although we can ask the proper questions of nature, we cannot always obtain a definite answer with present techniques. Others have not had Burk's logical scruples and have not hesitated to ascribe a specific role, for example, to molybdenum and many other inorganic ions (Burk and Burris, 1941, p. 605). Bortels (1930) first demonstrated that addition of molybdenum was necessary for maximum nitrogen fixation by Azotobacter, and this has been amply confirmed (Burk, 1934) and extended to the blue green alga *Nostoc*, that fixes N_2 (Bortels, 1936), the symbiotic system (Anderson, 1946; Bortels, 1937; Jensen and Betty, 1943; Jensen, 1946; Trumble and Ferres, 1946), and to the anaerobic nitrogen-fixing bacteria (Jensen and Spencer, 1946). The Australian workers, especially Trumble and associates, have demonstrated the practicability of supplying Mo together with other trace elements such as Zn to certain types of soil intended for the culture of legumes.

That the role of molybdenum in nature is not restricted to the nitrogen-fixers was evident when Steinberg (1936, 1937) demonstrated its necessity for utilization of nitrate by *Aspergillus niger*. Higher plants, both legumes and non-legumes, also respond to it under certain conditions (Arnon and Stout, 1939; Burk and Burris, 1941, p. 605). Nevertheless the view persists that it has a special significance for nitrogen fixation. On the basis of their experiments Burema and Wieringa (1942) decided that "for the assimilation of nitrate-N Azotobacter does not need as much Mo as is required for the assimilation of atmospheric nitrogen." Jensen arrived at the same conclusion for the symbiotic system in alfalfa and white clover, since the response to Mo added to sand cultures was considerably greater for plants fixing nitrogen than for the controls supplied $NaNO_3$, and since Mo accumulated in the nodules as compared with tops and roots (Jensen and Betty, 1943; Jensen, 1946). A note of caution, however, was sounded by Horner, Burk, Allison and Sherman (1942) who made a careful study of the Mo requirements of a number of strains of Azotobacter in specially purified media. Most strains of *Azotobacter chroococcum* fixed little nitrogen unless Mo was supplied up to 1 ppm, but strains of *A. vinelandii* fixed one-half to two-thirds their maximum in the absence of added Mo. That this arose from a more efficient use of traces in the purified medium appeared unlikely since the maximum fixation with *A. vinelandii* was also obtained only when the total Mo present approached 1 ppm. They concluded that undoubtedly Mo was required for the strains of *A. chroococcum* but whether it was essential for the others was questionable. Vanadium replaced Mo, although the final total nitrogen fixed was about one-third as great.

6. Hydrogenase. Cells of Azotobacter contain a powerful hydrogenase that catalyzes the reduction of methylene blue, oxygen, and other hydrogen acceptors by molecular H_2. Its properties such as response to inhibitors and pO_2 are

similar to those noted for the enzyme in other bacteria and algae (Wilson, Lee and Wilson, 1942; Lee, Wilson and Wilson, 1942; Wilson and Wilson, 1943). Its occurrence in Azotobacter is of special interest since H_2 acts as a specific inhibitor for nitrogen fixation. Lee and Wilson (1943) found that combined nitrogen not only inhibited assimilation of N_2 by Azotobacter but also decreased markedly its hydrogenase activity. For example, when *A. vinelandii* was cultured in a $H_2 + O_2$ atmosphere on combined nitrogen, the hydrogenase activity was low. Thus its elaboration by the organism appears to depend on the presence of N_2 rather than its specific substrate, H_2. Although the occurrence and functioning of hydrogenase in Azotobacter apparently is associated with

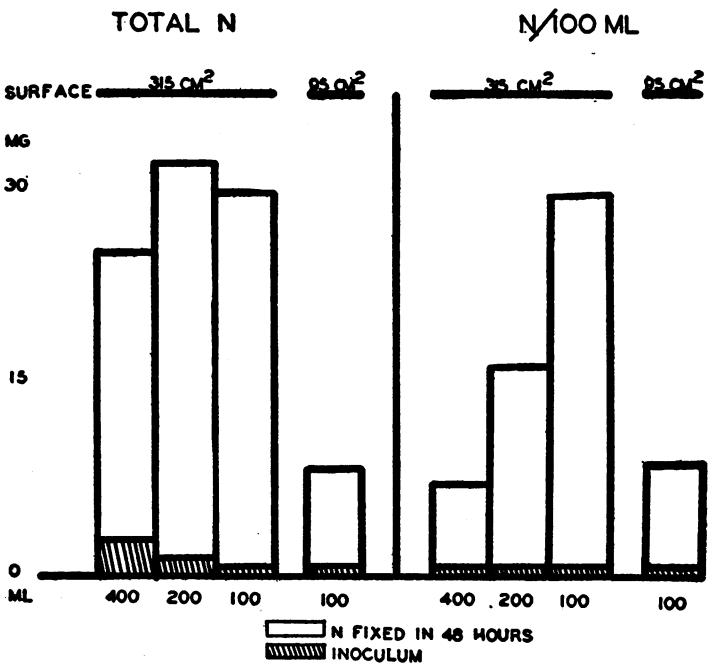

FIG. 2. Nitrogen fixation by *Azotobacter vinelandii* as a function of the surface of the medium (data of Wilson and Wilson, 1941)

nitrogen fixation, this does not appear to be true of the symbiotic fixation system in leguminous plants. The enzyme has not been found either in pure cultures of Rhizobium or in nodules from legumes (Wilson and Wilson, 1943; Wilson, Burris and Coffee, 1943).

7. *Role of Oxygen.* Nitrogen fixation by Azotobacter and by leguminous plants is usually stimulated by increasing the air supply. Typical results with Azotobacter shown in figure 2 illustrate that fixation is a function of the surface of the cultures, not of the volume. Similar results can be obtained by aeration (*cf.*, k values in Warburg respirometer with those in macro cultures) or even by the addition of about 0.1% of agar to the medium which apparently supports

a surface film. It is not yet established if the stimulation results from an increased supply of energy or has a more direct relationship to the fixation mechanism. Meyerhof and Burk (1928) and later Burk (1930) concluded from microrespiration experiments that the rate of nitrogen fixation reaches its maximum at a pO_2 of about 0.04 atm and that the efficiency (N_2 fixed/O_2 consumed) is maximum at about 0.01 atm. Since the effect of the pO_2 was independent of the source of nitrogen, Burk further concluded that the responses to O_2 cannot indicate the nature of the chemical mechanism of N_2 fixation. Wilson and Fred (1937) reported that, except possibly in the presence of high partial pressures of hydrogen, changes in the pO_2 caused similar effects in red clover plants whether they were using free or combined nitrogen.

The rôle of oxygen in fixation becomes of immediate interest because of the identification of the red pigment in the nodule with a hemoprotein similar to hemoglobin (Burris and Haas, 1944; Keilin and Wang, 1945; Kubo, 1939; Virtanen, 1945). Aside from the intrinsic significance of finding such a compound in plants, its function in N_2 fixation has caused varied speculation. Virtanen (1945, Virtanen and Laine, 1945a) suggests that with the accompanying methemoglobin it forms an oxidation-reduction couple based on a change of valence in iron that effects the reduction of N_2 to NH_2OH by a reversal of the reaction (Letsche, 1912):

$$\underset{\text{oxyhemoglobin}}{HbO_2\,[Fe^{++}]} + 2\,NH_2OH \longrightarrow \underset{\text{methemoglobin}}{HbO\,[Fe^{+++}]} + N_2 + 3H_2O$$

Aside from the fact that Letsche's characterization of the evolved gas was not unequivocal, this formulation is not in agreement with modern views of the reactions by which hemoglobin forms oxyhemoglobin and methemoglobin:

$$HbO_2\,[Fe^{++}] \rightleftharpoons O_2 + Hb\,[Fe^{++}] \overset{-e}{\rightleftharpoons} MetHb\,[Fe^{+++}]$$

Moreover, the reversal of Letsche's formulation implies that molecular oxygen is liberated along with the reduction of N_2 to NH_2OH. In presenting their suggestion, Virtanen and Laine, however, have substituted hemoglobin for oxyhemoglobin:

$$N_2 + \text{methemoglobin}\,[Fe^{+++}] \rightleftharpoons NH_2OH + \text{hemoglobin}\,[Fe^{++}]$$

$$\text{hemoglobin}\,[Fe^{++}] \xrightarrow{\text{oxalacetic acid}} \text{oxime} \longrightarrow l\text{-aspartic acid}$$

The rationale of this mechanism is puzzling since two oxidized compounds (N_2 and methemoglobin) are pictured as reacting to form two reduced compounds (NH_2OH and hemoglobin).[3] Finally it should be noted that the implication of

[3] Significant, perhaps, for the mechanism of the reaction studied by Letsche are the results of Warburg, Kubowitz and Christian (Biochem. Z., 1931 **242**, 170–205) dealing with the influence of phenylhydroxylamine on respiration by red blood cells. They found that this compound in the *presence of glucose* converted part of the hemoglobin (Hb) to methemoglobin (HbOH); an oxidation-reduction couple was thus formed that caused the rate of O_2

Virtanen, Laine and Linkola (1945) that the reduction of methemoglobin to hemoglobin in the nodules depends on the presence of oxalacetic acid is not supported by all the evidence. We have had no difficulty in finding the red pigment in nodules but have not been able to find detectable quantities of oxalacetic acid.

Virtanen and Laine (1945b) claimed fixation of N_2 in pure culture of *Rhizobium leguminosarum* if extracts of soybean nodules containing the pigment were supplied; the fixation was markedly increased if oxalacetic acid also was added. Later, Virtanen (1946) states that with more purified preparations of the pigment fixation was no longer obtained. Even so, if fixation in the presence of nodular extracts is confirmed, this discovery would be of great importance, as one of the most puzzling features of symbiotic nitrogen fixation is the absence of fixation by the bacteria apart from the host plant. During the summer of 1946 we tested more than 20 preparations of pigment from nodules of peas and soybeans in the presence of oxalacetic acid, α-ketoglutaric acid, citric acid and glucose but secured no fixation by the bacteria alone. Microrespirometer experiments (Little and Burris, 1947) demonstrated that addition of the pigment to cultures of bacteria including Rhizobium stimulated respiration at low pO_2 (0.01 atm). Because of the low pO_2 inside the nodule (Allison, Ludwig, Hoover and Minor,

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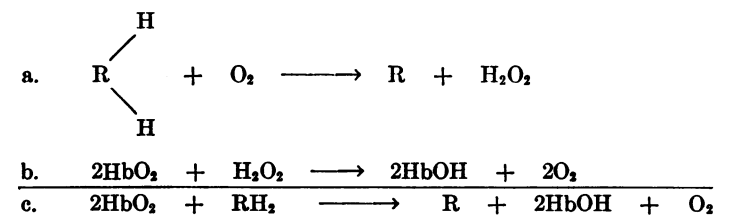
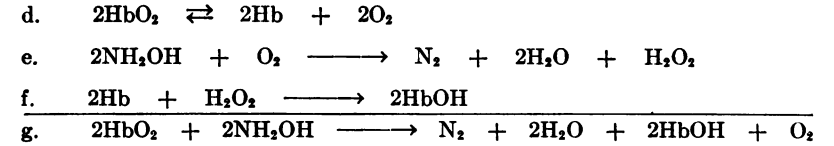
uptake to be increased 20 to 30 fold. The methemoglobin, reduced by substrate hydrogen, returned to hemoglobin but was reoxidized rather than oxygenated. From experiments in the presence and absence of CO and substrate, and from analogous reactions with phenylhydrazine and amyl nitrite, they concluded that the reaction was induced by formation of H_2O_2 (detected chemically) through auto-oxidation of the phenylhydroxylamine:



Although written as reacting directly with HbO_2, the results of trials with CO suggested that the actual reaction occurs with Hb in equilibrium with HbO_2. The net result is that the oxygen-binding capacity of the cells should be decreased twice that of the oxygen evolved. In an experiment, the observed ratio was 312/142 and the authors concluded that "Der Reaktionverlauf ist also nicht völlig klar."

If a similar reaction occurs with hydroxylamine and if, as seems probable from Letsche's results, the auto-oxidation of NH_2OH causes its decomposition, the series of reactions is:



Although reversal of the overall reaction might appear to be possible, consideration of the individual steps suggests that this is not a plausible mechanism for fixation of N_2.

1940; Frazer, 1942), it seems that a function of the pigment might be similar to that of hemoglobin in animals.

8. Fixation Systems in Different Organisms. An important corollary of defining the properties of the responsible enzyme systems in nitrogen-fixing organisms is that it establishes their similarities and differences. In general, the aerobic organisms capable of fixing nitrogen appear to possess enzyme systems remarkably similar as evidenced by their response to the pN_2, CO, H_2 and trace elements such as Mo. Establishment of this has been helpful for development and testing of proposed mechanisms, since each organism has certain advantages for different types of studies. Assurance that we can pool the information gained in studies with all the organisms should aid in final solutions of the many problems yet unsolved. Certain differences do exist, however, and these should be kept in mind as they too should be helpful in developing hypotheses of the mechanism. Among these are:

1. Hydrogenase has been reported only in the Azotobacter.
2. The hemoglobin-like pigment occurs only in the leguminous plant; although Azotobacter contains a red pigment, its bands indicate that it corresponds entirely to the cytochromes. Some cultures of *Azotobacter vinelandii* excrete into the medium a pigment that is variously pink and green dependent to some extent on the quantities of Fe and Mo present. It shows a wide nonspecific absorption band, and yields a blue derivative with pyridine.
3. Azotobacter has an extremely active aerobic respiration and requires a large amount of O_2, whereas *Clostridium pasteurianum* fixes N_2 anaerobically.
4. The rhizobia fix nitrogen only in association with the host plant; Azotobacter, *Cl. pasteurianum* and *Nostoc muscorum* fix nitrogen as free-living organisms.

POSSIBLE MECHANISMS OF FIXATION

For primarily descriptive purposes we can summarize the overall reactions that lead to fixation of N_2 in the following, not necessarily distinct, steps:

$$\text{Step I} \quad N_2 \rightleftharpoons N_2^*$$

$$\text{II} \quad N_2^* + E \rightleftharpoons N_2^*E$$

$$\text{III} \quad N_2^*E + X \rightleftharpoons N_2^*EX$$

$$\text{IV} \quad N_2^*EX \rightarrow E + N_2^*X$$

$$\text{V} \quad N_2^*X \rightarrow N_2X \text{ (Fixation)}$$

$$\text{VI} \quad N_2X \xrightarrow[\text{(intermediates)}]{\text{(unspecified)}} NH_3$$

$$\text{VII} \quad N_2^*X \rightarrow N_2^* + X \text{ (Decomposition without fixation)}$$

N_2^* is some activated form of molecular nitrogen, for example, a molecule possessing an energy, ϵ_a, greater than some critical ϵ_o. N_2^* is adsorbed on the

enzyme surface and reacts there with some unknown molecule, X; the combination is either deactivated, step V, which constitutes fixation, or may be decomposed without fixation, step VII. Alternatively, N_2^* may not be the sole form of N_2 adsorbed but is the sole form that takes part in the subsequent reactions.

With this formulation of the overall reaction in mind we realize the eventual necessity of defining more precisely steps III to VI. The situation is analogous to that faced by investigators of photosynthesis subsequent to Warburg's fundamental studies on the quantum relationships. Once the question was raised concerning the detailed steps in the photochemical and dark reactions, no mechanism could be seriously considered that offered no more than a mere restatement of the completed photosynthetic reaction. Although many false starts were made and cul-de-sacs explored, few would deny that progress toward understanding of this reaction began when experimental work was dictated by speculations regarding the detailed mechanisms and not just the overall process. We believe that a scientific maturity has been reached now in the study of biological nitrogen fixation that not only justifies but demands that we direct our attention to exploration of steps III to VI. This is particularly desirable since it emphasizes that enumeration of the descriptive steps does not properly constitute a mechanism although phrasing these in chemical symbols may give a deceptive appearance that such is being done. As a start we shall propose three typical schemes that we regard as possible mechanisms by which Azotobacter reduces N_2 to NH_3 and to suggest variations that might cover the symbiotic system. The purpose is not so much to establish which, if any of these, has the greatest probability based on information now available but to illustrate the type of problem that we believe should concern future investigations. A second purpose is to show through example how any proposed mechanism must be tested by the touchstone of whether it agrees with facts from all types of biochemical and physical-chemical investigations, not just the ones that suggested it. The story of the blind men and the elephant still has point for the scientific investigator.

Figure 3 outlines a general scheme which can serve as a working model for possible mechanisms in Azotobacter. It includes a hydrogenase playing an active rôle in the fixation process and provides a place for specific and competitive inhibition by hydrogen in the primary reaction leading to fixation. En_N is the specific enzyme that forms a complex with N_2 to enable it to react with the specific reductant (schemes I and II, see below) or oxidant (scheme III). En_H is the hydrogen-activating portion of the hydrogenase system and En_O the oxygen-activating. Yamagata and Nakamura (1938) concluded from experiments with inhibitors that hydrogenase is a specific enzyme that transfers H_2 to a common intermediate, A_h, which further reacts with the ultimate acceptor through one or more oxidation-reduction enzyme systems. The ultimate acceptor varies with the organism, but the hydrogenase system is the same for all. As the action of an inhibitor often is concerned with the transfer of H_2 from A_hH_2 to its ultimate acceptor, a given inhibitor does not always produce the same results in different organisms. For example, CN inhibits the Knallgas reaction in *Bacterium coli formicum* and *Rhodobacillus palustris* whose respiration

is cyanide-sensitive, but not in *Bacillus delbrückii* whose respiration is unaffected by cyanide. We have accordingly included an intermediate acceptor, A_h, and similar ones, B_o and B_o', (not necessarily different) for the oxidizing side of the reactions. Both A_h and B_o may represent none or several intermediary catalysts; that is En_H and En_O may be A_h or B_o, respectively, or these might represent a series of oxidation-reduction systems before reaching the reductant (or oxidant) specific for the primary reaction with $\{N_2\}$. Braces about a compound indicate that it exists as a complex with some other molecule, for example, with a specific enzyme although *initial* complex formation need not involve the specific enzyme indicated.

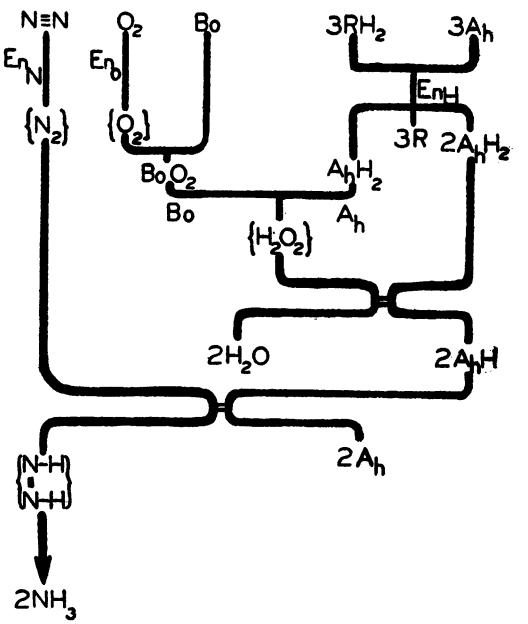


FIG. 3. Schematic representation of N_2 fixation by Azotobacter with substrate acting as hydrogen donor. See text for explanation of the symbols

Many of the symbols used in figure 3 as well as the arrangement of the reactions are based on Rabinowitch's (1945) discussion of various suggested mechanisms for reduction of CO_2 by green plants and bacteria. This was done not only because the device affords a compact summary of a great deal of information but also because we believe that evidence exists suggesting these two fundamental processes (reduction of CO_2 and N_2) may have certain points of similarity—for example, the possible rôle of hydrogenase in each. From the point of view of comparative biochemistry, then, it might be useful to employ similar basic patterns for postulated mechanisms.

Scheme I. The essential feature is that hydrogen for reduction comes from substrate (RH_2) for example, *via* the hydrogenase system. Whether hydrogenase functions in Azotobacter in this manner has not been experimentally

determined since it is not definitely assured that the organism normally evolves H_2. The older literature (Stoklasa, 1908) makes such claims, but purity of culture remains a question in these instances. Wilson and Wilson (1942) obtained evidence of the evolution of H_2 when masses of resting cells were anaerobically incubated with glucose and succinate, but the results were erratic. Production or transfer of H_2 does not necessarily require participation of hydrogenase, but because of its rôle in other organisms, it would be strange if it could not do likewise in Azotobacter. One mole of the reduced intermediate, A_hH_2, reacts with the oxidant to form the complex $\{H_2O_2\}$ which by reaction with 2 additional moles of A_hH_2 oxidizes the hydrogen of the latter to water with the formation of the free radical, A_hH. The free radical, containing the energy of the oxidation, brings about the first stage of fixation. The reactions may be summarized:

(1) $$3RH_2 + 3A_h \rightleftharpoons 3A_hH_2 + 3R;\ B_o + \{O_2\} \rightarrow B_oO_2$$

(2) $$A_hH_2 + B_oO_2 \rightarrow \{H_2O_2\} + A_h + B_o$$

(3) $$2A_hH_2 + \{H_2O_2\} \rightarrow 2A_hH + 2H_2O$$

(4) $$\{N_2\} + 2A_hH \rightleftharpoons \{N_2H_2\} + 2A_h$$

(5) $$\{N_2H_2\} \xrightarrow{\text{reduction}} 2\ NH_3$$

The key reaction is, of course, (4), and it would be here that H_2 would inhibit if competitive inhibition obtains. This could occur if molecular H_2 were to unite with the free radical, A_hH, to return it to its stable form A_hH_2, which is assumed to be incapable of reaction with $\{N_2\}$:

(6) $$H_2 + 2A_hH \rightarrow 2A_hH_2$$

After the initial fixed nitrogen compound $\{N_2H_2\}$ is formed, further reduction may occur through either A_hH or the more conventional oxidation-reduction systems (including A_hH_2) found in the organism. If we assume A_hH, steps 1 to 4 would have to be repeated twice more to effect the overall reaction for complete reduction to NH_3:

(7) $$N_2 + 3O_2 + 9RH_2 \rightarrow 2NH_3 + 6H_2O + 9R$$

If the 9 pairs of H_2 required are furnished by glucose, the reaction for fixation becomes:

(8) $$N_2 + 7.5O_2 + 1.5\ \text{glucose} \rightarrow 2NH_3 + 6H_2O + 9CO_2$$

Equation (8) suggests that the ratio, moles N_2 fixed per mole O_2 used could equal 0.133; Meyerhof and Burk (1928) from thermodynamical considerations estimated this as having a maximum value of 0.915. In their experiments the ratio varied from 0.003 to 0.008 in air to 0.10 in an atmosphere with a pO_2 of 0.0012 atm.

Scheme II. A second possibility is that the hydrogen for reduction comes from a reversal of the Knallgas reaction; although this might appear to be

difficult because of the large value of $\Delta F^\circ = 56.6$ kg cal, it could proceed readily enough if it were part of an oxidation-reduction reaction:

$$H_2O + A_h + B_o' \rightarrow A_hH + B_o'(OH) \quad (9)$$

In equation 9 the decomposition of water is written so as to provide immediately for creation of free radicals; this is mainly for simplification, as it may be more consistent to expect first the formation of the stable forms (A_hH_2 and $B_o'(OH)_2$) followed by creation of free radicals by oxidation-reduction reactions as in scheme I. Not only does this proposal provide a definite rôle for hydrogenase, but it furnishes a new unity in biochemistry through merging of photosynthesis and nitrogen fixation. Most authorities agree that the reduction of carbon dioxide by green plants is accomplished by hydrogen generated from the splitting of water (Rabinowitch, 1945, chap. 7); many would extend this concept to bacterial photosynthesis (Gaffron, 1940; Nakamura, 1937; van Niel, 1941). Gaffron's (1942) fundamental discovery that certain green algae can couple the reduction of CO_2 with the oxyhydrogen reaction, and thus dispense with the light reactions, is suggestive for nitrogen fixation.

In Azotobacter the energy for the splitting would probably come from energy-rich ~P bonds generated by respiration (*cf.*, Lipmann and Tuttle, 1945); molecular nitrogen would act as the hydrogen acceptor instead of CO_2; and the oxidizing portion of the system ("hydroxylated" enzyme), would be regenerated through reaction with substrate hydrogen. Inhibition by H_2 would occur as in *Scheme I* at reaction 4.

Scheme III. Alternatively, the primary reaction involving N_2 might be its oxidation by means of $B_o'(OH)$:

$$N_2 + 2B_o'(OH) \rightleftharpoons \left\{\begin{matrix} N{-}OH \\ \| \\ N{-}OH \end{matrix}\right\} + 2B_o' \quad (10)$$

If so, it suggests that inhibition of N_2 fixation by nitrate (nitrite) occurs through formation of some intermediate such as $\left\{\begin{matrix} N{-}OH \\ \| \\ N{-}OH \end{matrix}\right\}$ rather than the ultimate product of reduction, NH_3. That is, N_2 assimilation joins the metabolic pathway of NO_3^- assimilation at an early stage; hence fixation is inhibited by nitrate when reduction of NO_3^- proceeds at a rate sufficiently rapid to maintain a constant supply of the common intermediate. Certain similarities in the response of Azotobacter when furnished NO_2^- and NO_3^- to that obtained with N_2, *e.g.*, to CO (Lind and Wilson, 1942; Wilson and Lind, 1943) provide some support for this view. It should be noted that just as $\left\{\begin{matrix} N{-}H \\ \| \\ N{-}H \end{matrix}\right\}$ implies only reduced nitrogen, $\left\{\begin{matrix} N{-}OH \\ \| \\ N{-}OH \end{matrix}\right\}$ represents only oxidized nitrogen.

GENERAL EXAMINATION OF THE HYPOTHESIS

In the preceding section we have proposed three possible schemes for N_2 fixation suggested by certain experimental results. Others could undoubtedly be

offered, but these include interesting possibilities and, though not exhaustive, they certainly are typical. It remains to examine these in terms of all information we have on the biochemistry and biophysics of the process. This is essential not only because even tentative acceptance depends on demonstration that the implications do not conflict with available information but also because such examination suggests necessary revision and future experiments. In this section general considerations will be explored with indications of what type of experiment might provide critical data; the following section will discuss details of the reactions.

Thermodynamics. The proposed schemes appear to be thermodynamically sound. Reduction of N_2 by metabolic H_2 can be written as a first approximation:

(11) $0.5\ N_2 + 1.5H_2 + H_2O \rightleftharpoons NH_4^+ + OH^-;\ \Delta F^\circ = 0.17$ kg cal

At pH 7.0 this reaction can be represented by the following energy equation based on the convention that we are dealing with an oxidation-reduction system, N_2/NH_4^+:

(12) $$E = -0.28 + \frac{RT}{3F} \ln \frac{(\sqrt{N_2})}{(NH_4^+)}$$

At a pN_2 of 0.8 atm and (NH_4^+) equal to 10^{-4} M, E becomes -0.20 v. To determine if "average" metabolic hydrogen, for example that in glucose, could bring about this reduction, we write the following series of reactions which summarize the energy changes to be calculated.

It is emphasized here, since many authors of bacteriological texts fail to make this distinction, that these and similar equations do not represent actual mechanisms but merely convenient formulations which are useful for thermodynamical calculations. Such calculations are largely independent of mechanism but are concerned only with initial and final states. Since these are primarily energy and not chemical equations, for completeness they require specification of conditions and concentrations. Some can be assigned without question, whereas others demand estimates or even guesses. Our choices in these instances have not been entirely arbitrary, however, but are based on the best information available. The pCO_2 is taken at 0.01 atm instead of the traditional 0.0003 atm as the former is more in harmony with the actual concentration of CO_2 over quiescent or even agitated cultures. The concentration of ammonium, 10^{-4} M, is based on residual levels of ammonium when this source of nitrogen is added as well as on excreted metabolic NH_4^+-N found in young cultures fixing N_2 (Horner and Burk, 1939). The CO_2 liberated will appear as both free gas and bicarbonate ion at pH 7; theoretical proportions of each are indicated in the equations. These were calculated from the pK_1 of CO_2 (aq) together with the (HCO_3^-) in equilibrium with a pCO_2 of 0.01 atm at that pH.

(13) $C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2$

(14) $4N_2 + 12H_2 + 8H_2O \rightarrow 8NH_4^+ + 8OH^-$

(15) $6CO_2 + 4.67H_2O \rightarrow 4.67HCO_3^- + 4.67H^+ + 1.33CO_2$

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(16) $C_6H_{12}O_6$ (0.05 M) + 4 N_2 (0.8 atm) + 18.67 H_2O (l) $\rightarrow$
8 NH_4^+ (10^{-4}M) + 8 OH^- (10^{-7}M) + 4.67 HCO_3^- (0.0012 M) +
4.67 H^+ (10^{-7}M) + 1.33 CO_2 (0.01 atm)

Although the standard free energy of this reaction (ΔF°) is +5.6 kg cal per 0.5 N_2, the organism actually obtains energy by carrying out the reaction under the specified conditions, ΔF equalling −17.5 kg cal per 0.5 mole N_2 fixed. In ordinary laboratory experiments we can assume that Azotobacter will assimilate about 150 mg of 1,000 mg glucose utilized and will fix approximately 14 mg N_2 (Lee and Burris, 1943). The energy balance for 1,000 mg of sugar is:

150 mg assimilated	Small, if any, change in free energy
22.5 mg used in N_2 fixation	17.5 cal released
827.5 mg oxidized	3,200 cal released

As can be seen from these values, the energy loss is extremely small and probably undetectable in ordinary experiments if the organism substitutes N_2 for O_2 as the acceptor of part of its metabolic hydrogen. Usually, the organism has available about 98 per cent of the energy that it would obtain if NH_4^+−N were supplied so that it could oxidize completely the 850 mg glucose used in forming 150 mg of cell material.

Similar conclusions are reached if we assume that the H_2 used for reduction comes from water exclusively rather than from the substrate (*Schemes II and III*). If the splitting of water is assumed to be: $H_2O \rightleftarrows \{H\} + \{OH\}$, three moles would be required for the reduction of 0.5 mole of N_2. Since the O-H bond is equal to 110 kg cal (Rabinowitch, 1945), approximately 30 high energy ~P bonds would be necessary. These are roughly equivalent to the oxidation of one mole of glucose; hence one gram of glucose would be sufficient for fixation of about 80 mg of nitrogen, whereas actually 10 to 15 mg is usually fixed. The energy balance, however, is probably much more favorable since accompanying oxidation-reduction reactions, for example by the {OH}, could be coupled with production of ~P bonds.

Another method of approach to the energetics of the process is estimation of the energy required to form the activated complex that is assumed to govern the rate of the enzyme-catalyzed reaction (Moelwyn-Hughes, 1933, 1937; chapter 10 in Elvehjem and Wilson, 1939). Even though precise evaluation is not yet possible, an idea of its magnitude can be obtained from molecular statistics of the process. If the Azotobacter is a sphere 2 μ in diameter, it has a volume of 4.2×10^{-12} cm^3, a surface area of 12.6×10^{-8} cm^2, and 6.3×10^{-14} g organic nitrogen (1.5 per cent N) equal to 1.4×10^9 molecules of nitrogen. Assuming that En_N has a diameter comparable to that of other enzymes, about 5×10^{-7} cm, we consider that each enzyme molecule would occupy an area of 25×10^{-14} cm^2 and therefore the surface of each cell could accomodate as many as 0.5×10^6 enzyme molecules. One ml of medium containing 0.15 mg bacterial nitrogen would represent 2.5×10^9 cells. The number of effective collisions between N_2 in solution and enzyme molecules can be estimated from

$$Z = n_1 n_2 \frac{(\sigma_1 + \sigma_2)}{2} [8 \pi RT (1/M_1 + 1/M_2)]^{1/2} \sigma_1^2/\sigma_2^2 \quad (13)$$

in which

n_1 = number of molecules of N_2 in 1 ml, 7.3×10^{16} at a pN_2 of 0.2 atm
n_2 = number of enzyme molecules in 1 ml, 1.25×10^{15}
σ_1 = diameter of N_2 molecule, 3×10^{-8} cm
σ_2 = diameter of enzyme molecule, 5×10^{-7} cm
R = 8.3×10^7 ergs/degree
T = 304°K.
M_1 = 28; M_2 is probably at least 1000 times M_1, hence $1/M_2$ can be neglected
$\sigma_1^2/\sigma_2^2 = 3.6 \times 10^{-3}$ measures the probability that a collision will be effectively oriented.

When these values are substituted in (13), $Z = 3.5 \times 10^{21}$ collisions sec^{-1}. The specific rate constant k is 0.4 hr^{-1} or 1.1×10^{-4} sec^{-1} which means that in the first second of reaction the number of N_2 molecules fixed is:

$$(1.1 \times 10^{-4})\ (0.15 \times 6.06 \times 10^{23})/28{,}000 = 3.6 \times 10^{14}$$

$$\text{Then } \frac{3.6 \times 10^{14}}{3.5 \times 10^{21}} = e^{-\Delta E/RT} = e^{-16.1} = 10^{-7}$$

$$\Delta E = 10 \text{ kg cal}$$

A second method of approximation that might be applicable is to regard the reaction as bimolecular between N_2 and enzyme molecules as Moelwyn-Hughes (1937) illustrates with decomposition of H_2O_2 by catalase.

$$(14) \qquad k_{bi} = -dn_1/dt \times 1/n_1n_2 = \sigma_1^2 \sqrt{\frac{\pi k' T}{2m_s}}\ e^{-\Delta E/RT}$$

k′ = Boltzmann's constant, 1.37×10^{-16} erg/degree

$m_s = 28/(6.06 \times 10^{23})$

Since in Azotobacter the number of enzyme molecules is constantly changing unimolecularly and the number of N_2 molecules in solution may be regarded as constant, the bimolecular constant may be estimated by dividing the calculated unimolecular constant by n_1; substituting in (14), $\Delta E = 13$ kg cal. Burk (1934, p. 40) who used still another formula for calculating the number of collisions arrived at values for ΔE of 11.7 to 17.5 kg cal.

The major uncertainty, other than the pertinency of a given formula, is the number assigned for enzyme molecules per ml; the value assigned to σ_2 is relatively ineffective in (13) and disappears from (14). That the estimate of n_2 is reasonable is indicated by calculation of the *turnover number* (TN), the number of molecules of nitrogen fixed per molecule of enzyme per minute. Since 3.6×10^{14} molecules of N_2 are fixed per sec. by 1.25×10^{15} molecules of enzyme, the turnover number is 18. This is rather low in comparison with the TN of enzymes that have been studied in purified state (Green, 1940; Green, Leloir and Nocito, 1945; Sumner and Somers, 1943) so that our estimated number of enzyme molecules per cell is more likely to be too high than otherwise.

Confirmation for this view is supplied by the experiments of Lineweaver (1938) who attempted to measure directly the hyperbolic (chemical or physical) adsorption of N_2 indicated by the kinetic studies (Lineweaver and Burk, 1934; Lineweaver, Burk and Deming, 1934; Wilson, Burris and Lind, 1942). No evidence of such adsorption was obtained, the results being explicable on the basis of Henry's solubility law. It was concluded that the adsorption was beyond the limits of the method, which set an upper limit of 0.04 to 0.4 $\times$ 10^6 to the number of N_2 molecules adsorbed per cell. Every 10- fold decrease in the number of enzyme molecules means a 1.6 kg cal decrease in ΔE; therefore, an activation energy considerably more than about 12 kg cal, or about one $\sim$P, does not appear probable.

A second type of "turnover number" can be calculated if it is assumed that only those molecules react that possess sufficient energy by reason of thermal activation. Approximately one molecule in 10^7 possesses sufficient energy and since about 0.3 molecule is fixed per sec. by each molecule of enzyme, it would have to adsorb between 10^6 and 10^7 molecules of N_2 per sec. to account for the observed rate of reaction. Although not a true TN, this value is similar in that it measures the rate of "combination" and "decomposition" of N_2 and En_N. Decomposition of H_2O_2 by catalase is about the only known enzyme system with such a high TN; hence it appears likely that activation of the N_2 molecule depends on enzyme intervention rather than the fortuitous distribution summarized in the Maxwell-Boltzmann law.

Comparative Biochemistry. Probably the most important biochemical fact established by the physical-chemical studies has been the unity among the N_2 fixation processes in Azotobacter, the blue-green alga, *Nostoc muscorum*, and the symbiotic system. The mechanisms suggested in the preceding section were based on the studies with Azotobacter, and one of the most critical tests is whether they describe equally well fixation by the other organisms. Certain variations would be expected because of the differences already noted among the systems, but in general the basic pattern should be the same. The first point of consideration is whether it is essential that ammonia be the key intermediate as postulated in these schemes. Obviously this is not a critical part of any of the proposals so that decision on this point will affect their validity but little. Although recent studies definitely suggest ammonia as the key intermediate in fixation by Azotobacter, it is not claimed that these rule out NH_2OH in the symbiotic system. Our view, first stated in Wilson (1940, p. 184) and more recently affirmed (Burris and Wilson 1945, p. 700), that in the symbiotic system both intermediates may function, depending on the particular keto-acids or similar acceptor available, still appears to be valid.

But to state this possibility does not imply that it has been experimentally established. Before this could be accepted, studies similar to the ones already made with Azotobacter and the heavy nitrogen isotope would be necessary. It must be demonstrated that the system of plant plus bacteria can use NH_2OH or at least suitable oximes and that accumulation of tagged nitrogen during fixation is similar to that observed with these and not to that with NH_4^+. Similar

studies with Azotobacter should be undertaken since at present even the minimum requirement—that of utilization—is denied (Burk and Burris, 1941, p. 601). Corroboration of the excretion data (Wyss and Wilson, 1941b), especially the identity of the oxime found in small quantities (Virtanen and Laine, 1939), would be helpful although not as impressive as the metabolic tracer studies. The isotope dilution technique (Rittenberg and Foster, 1940; Rittenberg, in Green, 1946) has been useful in analogous problems and might be employed here. A better understanding of conditions necessary for occurrence of both excretion and fixation by excised nodules so that more consistent results can be obtained likewise may prove of significance for the rôle of NH_2OH. Finally, a detailed study of the rôle of organic acids in the intermediary metabolism of leguminous plants is essential.

Of interest and possibly of significance for the biological problem are the results of chemical studies on nitrogenous compounds. Nichols and Derbigny (1926) report that reduction of N_2O proceeds quantitatively to N_2, NH_2OH or NH_3 dependent on the oxidation-reduction potential of the reducing system used and that no mixture of products occurs. Joss (1926) summarizing similar work on reduction of nitrate, nitrite, and other oxides of nitrogen concluded that NH_2OH probably represents a side reaction in the formation of ammonia rather than an immediate precursor. These chemical studies emphasize that, although NH_2OH may appear in the reduction of N_2 to NH_3, its occurrence is not a necessity.

An even more serious lack of knowledge concerned with the comparative biochemistry of the process arises from the few studies of the mechanism made with Nostoc and still fewer with Clostridium. Investigations with the anaerobe are especially desirable now that the nutritional requirements of organisms are better understood so that more rapid fixation than has been obtained in the past should now be possible. The rôle of hydrogen and hydrogenase in the aerobic processes makes imperative that we learn more details of the anaerobic counterpart.

SPECIFIC EXAMINATION OF THE HYPOTHESES

Schemes I and II. The next step is to decide whether details of any of the schemes conflict with known experimental facts or reasonable assumptions about the fixation process in the various agents. The first two schemes may be considered together, as scheme II is in one sense only a special case of scheme I. Since hydrogenase has not been found in Rhizobium, nodules, or Nostoc, the reducing system for these is not hydrogenase (scheme I); and scheme II is restricted to Azotobacter. The possibility remains that, as a special case of scheme II in the plant systems, hydrogen from water split in the photosynthetic reaction rather than from reversal of the Knallgas reaction acts as the reductant. Lacking direct experimental support, this appears unlikely, and in the leguminous plant transfer of the reducing system from the chloroplasts to the nodules offers formidable difficulties. The participation of hydrogenase, however, is essential for scheme II only, as any other reducing system with the proper potential could

function in scheme I. There is no *a priori* reason why the reducing system in the various agents should be identical; in algae not only does the hydrogen-donating system for reduction of CO_2 vary among closely related species but even in the same species at different times (Gaffron, 1944). Whether hydrogenase actually is the reducing system in Azotobacter as postulated awaits further clarification of the hydrogen metabolism of the organism, but present evidence suggests it as a likely possibility.

A point much more critical is the necessity for introducing the free radical mechanism in these schemes. In photosynthesis this appears desirable to provide a system with sufficient energy to reduce CO_2 (Rabinowitch, 1945, chap. 9). Equation 12 suggests that the assumption for N_2 fixation is gratuitous as many of the conventional oxidation-reduction systems possess potentials sufficiently low to function. However, the fact that a reaction is thermodynamically feasible does not necessarily mean that it will proceed at a measurable rate. It is possible that a highly active complex is needed to surmount an initial high energy potential even though the overall reaction is downhill (see discussions by Michaelis and by Kalckar in Green, 1946). However, the estimates of the activation energy made in the preceding section are hardly suggestive of an unusually difficult energy barrier.

Apart from considerations of energetics, the chief advantage of a free radical mechanism for N_2 fixation is that it provides a definite place for *competitive* inhibition by H_2. If reduction is accomplished by metabolic hydrogen involving only normal molecules and conventional oxidation-reduction systems, A_hH_2 does not appear to be a plausible reducing system since the presence of H_2 should provide another hydrogen donator; hence inhibition by H_2 is unexpected. If one tries to surmount this difficulty by reasonable postulates, an almost inevitable consequence is that inhibition by H_2 should be non-competitive.

As an alternative we can assume that reduction is carried out by some conventional respiratory system and that H_2 inhibits primarily by mechanical blocking of the N_2 molecule from the surface of En_N. If so, van der Waals' forces might serve as a rough measure of the relative adsorption of the two molecules. For H_2 and N_2 this is $0.00277/0.000487 = 5.7$ which is unexpectedly close to the ratio of their Michaelis constants of 5.5. A corollary would be that other gases should inhibit. Of those tried CO and O_2 are ruled out because of accompanying physiological effects, and the van der Waals constant for helium, 0.00007, appears too low for detection. Argon with a constant of 0.00268 should be even more effective than H_2; but experimentally no inhibition is obtained with argon. This point should be tested further with other gases as it has attractive possibilities, though, unless H_2 has a specific action, the rôle of hydrogenase appears vague and uncertain.

Scheme III. The main advantage of this scheme is that it avoids the necessity for postulating a free radical mechanism. Although written in equation (10) as if one were involved, this is an unnecessary assumption since H_2 could compete with N_2 for the "normal" state of the oxidant, B_o' $(OH)_2$, ("hydroxylated" enzyme) taking part in the initial fixation step (*cf.* Gaffron, 1940). Its

chief disadvantage is lack of relevant experiment. Clarification of the pO_2 function of Azotobacter in the presence and absence of H_2 and the rôle of the pigment system in the nodule should be helpful in deciding whether an oxidation is a probable first step in fixation.

If an oxidation step appears likely, identification of the initial oxidized product is of interest. As written in the equations, hyponitrite is suggested. Many investigators have postulated this compound as an intermediate in reduction of nitrates (nitrites) to ammonia or free nitrogen and in oxidation of ammonia to nitrous acid by various bacteria. Sufficient experimental support exists to make some of these claims probable (Corbet, 1935; Elema, Kluyver and van Dalfsen, 1934; Lloyd and Cranston, 1930). If it is an intermediate in the reduction of nitrate by Azotobacter, it meets the first requirement of an intermediate for N_2 fixation, since obviously it is assimilable. Steinberg (1939) reports that hyponitrite gives a positive test with Blom's reagent for NH_2OH; hence the positive tests for this compound could be interpreted equally well as evidence for HNO.[4] Opposed to this evidence is the observation of Wilson and his collaborators that N_2O, supposedly the anhydride of HNO, cannot serve as a source of nitrogen for the Azotobacter. Until this very critical requirement is met, HNO must be rejected for the same reason as was NH_2OH.

SUMMARY

Ammonia emerges as the most likely key intermediate in biological nitrogen fixation, with strong experimental support based upon studies of the properties of the enzyme system and upon observations with N^{15} as a tracer. The evidence has been gathered chiefly with Azotobacter, and it might be held dangerous to attribute the same properties to all biological nitrogen-fixing systems; nevertheless, until more critical evidence is presented for the hydroxylamine hypothesis, it seems justifiable to assign a general rôle to ammonia.

A generalized scheme for nitrogen fixation has been presented in figure 3, and from it three specific routes of fixation have been proposed and examined. The first assumes reduction of nitrogen with substrate hydrogen and on the whole seems the most likely pathway of fixation. However, certain objections can be raised to it, and scheme II, with a reversal of the Knallgas reaction furnishing the hydrogen for reduction, and scheme III, with an oxidative mechanism, should be considered as possible alternative routes and subjected to experimental test.

Although cause for satisfaction exists in considering the progress made toward an understanding of the mechanism of biological nitrogen fixation during the past decade, this review emphasizes the need for additional critical information. The state of our knowledge recalls Kluyver's (1931) comment of a similar period in the development of bacterial respiratory mechanisms.

[4] Endres (1935) claimed that HNO could not be among the products giving a color with Blom's reagent in cultures of Azotobacter as he had boiled these overnight in acid solution. The evidence is equivocal, however, since he recovered only 10 per cent of the material responsible for the color after such treatment.

"... the evidence for the occurrence of a special intermediate stage in a biochemical process *can* only be of an indirect nature. From this point of view it follows that one can only judge the probability of a supposed reaction mechanism by co-ordinating all experimental data available."

"... either we have to accept the fact as it is and refrain from any explanation, or we have to tread the path of speculation and test the probability of different hypotheses as to the nature of this mechanism."

The special steps we have proposed in this review describe more or less accurately laboratory results based on a necessarily small sample of the totality of experimental facts to be gathered eventually. Unfortunately, by reason of the experimental approach such a sample often fails to represent the random choice desired. As suggested specifically in the text, further samplings with the methods that have proved so useful in recent work together with newly developed ones should provide a basis for choice among the suggested mechanisms, demand their modification or even rejection.

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