CCLXIX. MECHANISM OF SYMBIOTIC NITROGEN FIXATION

IV. SPECIFIC INHIBITION BY HYDROGEN[1]

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(*Received 23 September 1938*)

BECAUSE of the practical importance of the problem in agriculture, research on the fixation of atmospheric nitrogen through association of the root nodule bacteria and leguminous plants has been primarily directed towards answering questions of immediate agronomic significance. As a result, advances concerned with the more fundamental aspects of this biological process have lagged behind practical applications. Part of the lag, however, may be ascribed to lack of suitable techniques for investigation of the biochemical phases of the problem. In symbiotic nitrogen fixation the life processes of two organisms, plant and bacteria, are intimately related so that there is great difficulty in isolating the factors which have special significance for the fixation reaction. This difficulty has been overcome in part by Wilson and collaborators [Wilson, 1936; Wilson & Fred, 1937; Wilson & Umbreit, 1937] through the application of a physico-chemical approach to the study of the properties of the enzyme system responsible for the fixation.

In the initial studies, which were undertaken to determine the influence of the pN_2 in the atmosphere on the fixation reaction, it was observed that the partial pressure of N_2 could be lowered to about 0·10–0·15 atm. without decrease in the quantity of N_2 fixed by nodulated red clover plants provided that the N_2 removed was either unreplaced or replaced with helium or argon. If the N_2 were replaced with H_2, however, the total quantity of N_2 fixed decreased linearly with the pN_2 of the atmosphere throughout the range 0·06 to 0·80 atm. As such a finding suggests that H_2 may be a specific inhibitor for the symbiotic N_2 fixation process, detailed studies of the "hydrogen effect" were undertaken.

Of primary importance is the demonstration that the effect of H_2 on the assimilation of free N_2 is quantitatively different from the effect on the uptake of combined forms, as NH_4^+ and NO_3^-. A previous report [Wilson & Umbreit, 1937] discussed the various types of experiment which might be expected to throw light on this question; in the same report data were summarized from experiments in which the *total quantity* of N_2 fixed was used as a measure of the effect of H_2. In this paper other data will be presented from experiments in which the *rate* of fixation is used as a criterion.

Methods

Twenty red clover plants (*Trifolium pratense*, L.) were grown in a N-poor sand substrate placed in 9-l. pyrex pressure bottles. All plant nutrients except combined N were added to the sand. Plants of one series were inoculated with an effective strain of the specific organism, *Rhizobium trifolii*; plants of a second series were not inoculated but furnished periodically with combined forms of N.

[1] Herman Frasch Foundation in Agricultural Chemistry, Paper No. 149.

Under the conditions of the experiments the limiting factor for development of the plants was the supply of N. Each bottle was closed with a gas-tight rubber stopper fitted with connexions for changing the atmosphere given the plants. Details of the methods have been described previously [Wilson, 1936].

General considerations of the experiments

As was emphasized in an earlier paper [Wilson & Umbreit, 1937], rate experiments with plants grown in a controlled environment present technical difficulties associated with restriction of available space and apparatus. In most experiments there were six treatments (three different partial pressures of H_2 with two series of plants); at each harvest cultures from each treatment were harvested in duplicate. Since in this work not more than about 50 plant cultures could be made in any one experiment, the maximum number of harvests which could be taken was four. The period during which the plants were under different atmospheres rarely exceeded a month; at the end of this time the inoculated plants in air (or plants which had been given combined N) completely filled the plant container. As it was inadvisable to make a harvest more frequently than once a week, this consideration once again imposed a limitation of four harvests.

When the quantity of free or combined N assimilated by red clover during the period under study is plotted against time, the resulting curves are logarithmic in form; when plotted on semi-log paper the points fall along a straight line. The following factors will cause minor deviations from this straight line.

(1) *Sampling errors.* In spite of all efforts to maintain the plants in identical environments other than the composition of the atmosphere furnished them, variations between the N content of replicates reached 10–15%. Statistical analysis of a large number of experiments [Wilson, 1936; Wilson & Umbreit, 1937] indicates that the *mean* of duplicate samples has a standard error of 5 to 8%. In rate experiments if only duplicate samples are taken at each harvest, through chance these may be cultures with N content definitely lower (or higher) than the mean of all the samples of that particular treatment. An inaccurate sample at one harvest will affect succeeding samples in an opposite direction since the remaining samples will be higher (or lower) than the general mean. For this reason it is believed that the best straight line which can be drawn through the experimental points probably represents the true course of development of the plants. In calculations, points on this line are taken rather than the actual values derived from the samples.

(2) *Restriction of growth.* As has been mentioned, at the end of the experiment the plants in certain of the treatments almost fill the container. During the last week of growth it is probable that the development of these plants is restricted to some extent because of competition for space. This restriction will cause the observed value for N uptake to be less than it would have been had the competition not occurred. A partial remedy for this may be to grow fewer plants in the container, but a reduction in the number of plants taken for analysis will increase the sampling error so that little is gained. Moreover, a great deal of the restricted growth obtains because the plants have reached the top of the container and are more easily shaded by the stopper and other attachments on the bottle. Decrease in the number of plants per bottle would not correct this cause of restricted growth.

(3) *Change in environment.* In spite of the fact that the experiments are relatively short-time in nature, during certain seasons there may be encountered great variations in the weather which will affect the rate of development of the plants for short time intervals. Little can be done to avoid the vagaries in the climate other than to perform the experiments, in so far as is convenient, during seasons of fairly stable weather conditions.

If the development of the plants is logarithmic with respect to time, it is advantageous to use the specific constant of N assimilation, g, for comparison of effect of treatment [Burk, 1934]. This constant is defined by

$$g = 2{\cdot}303 \frac{d \log (a+y)}{dt} = 2{\cdot}303 \times \text{slope straight line of semi-log graph}$$

in which a equals N content of plants at start of treatment and y equals increase after time t. The value of this constant represents rate of assimilation of N per unit concentration of N and is therefore independent of the time.

Rate experiments

Two experiments have been made in which four harvests were taken, and three others in which there were three harvests. In the latter experiments another aspect of the problem was under simultaneous investigation (see next section) so that not all the plant cultures were available for the simple rate tests.

Exp. 1. This was made during the early autumn months, and the natural illumination was supplemented by artificial light. NH_4NO_3 was used as the source of combined N. The assimilation of N is shown in Fig. 1. From these data it is evident that the inhibition of N fixation by H_2 does not arise from mere restriction of fixation during the early stages of treatment but is an effect which persists throughout the experiment. In contrast to this effect of H_2 on the assimilation of elemental N the uptake of combined N is independent of the pH_2 in the atmosphere.

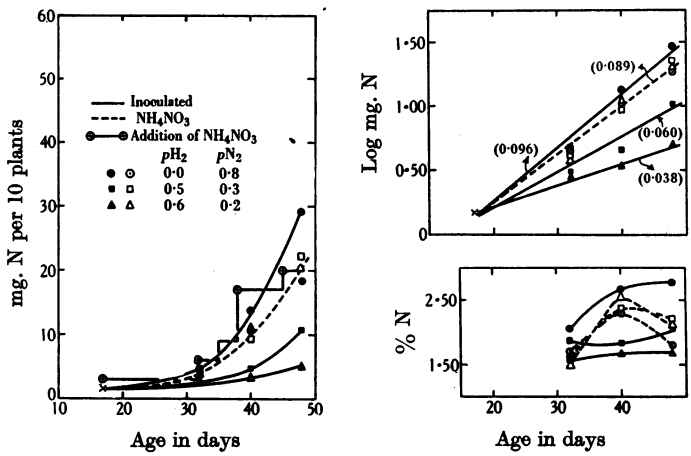

Fig. 1. Comparison of assimilation of free and combined N (NH_4NO_3) by red clover in atmospheres of various pH_2. Figures in parentheses represent g values.

(Exp. 1, planted 12 September; all cultures in air until harvest I, 29 September, then changed to atmosphere indicated; harvest II, 14 October; harvest III, 22 October; harvest IV, 30 October, 1936.)

When log mg. N is plotted against time, the fit of the points to a straight line is quite satisfactory except possibly of those for the atmosphere containing a pH_2 of 0·4 atm. Even in this case the deviations from the line hardly exceed sampling errors. The points corresponding to the plants given combined N fall along a single line, so the g values are identical for these plants whether H_2 is present or not. In contrast, the g values for plants dependent on the fixation of free N decrease with an increase in the pH_2 of the atmosphere.

The effect of H_2 on the % N in the plants should not be overlooked. With *inoculated plants* there is a marked decrease in the % N with increase in the pH_2 of the atmosphere. This means that N fixation is so restricted in these plants when grown in the presence of H_2 that carbohydrates accumulate. The appearance of the plants reflects this difference in composition; plants grown in air are

dark green in colour whereas those grown in an atmosphere containing H_2 have yellow leaves and red stems. With plants given combined N there is no association of % N with the pH_2, and the plants are indistinguishable in general appearance.

It is emphasized that the effect of H_2 on the N fixation process does not arise from the simultaneous decrease in the pN_2 of the atmosphere since in the range used the rate of fixation is independent of pN_2 [Wilson, 1936].

Exp. 2. As this was done during the summer months, light and other weather conditions were ideal for N fixation. The roof of the greenhouse was equipped with a water cooling system, and the bottles were kept in a trough of flowing water in order to avoid excessive temperatures. Under these conditions the development of the plants was most satisfactory. In Exp. 1, through control of the addition of NH_4NO_3, the rate of assimilation of combined N was kept nearly equal to the rate of assimilation of free N_2 by the inoculated plants. The objection could be raised that such a procedure might mask an inhibition by H_2 of the uptake of combined N. If some other factor, such as supply of combined N, is restricting the growth of the plants, inhibition by H_2 might not be obvious. In Exp. 2 $(NH_4)_2HPO_4$-N was always supplied in excess so that the rate of uptake was not restricted because of low concentration of available combined N. $(NH_4)_2HPO_4$ was used, as other experiments had indicated that development of clover was less erratic with this form of N [Wilson & Umbreit, 1937].

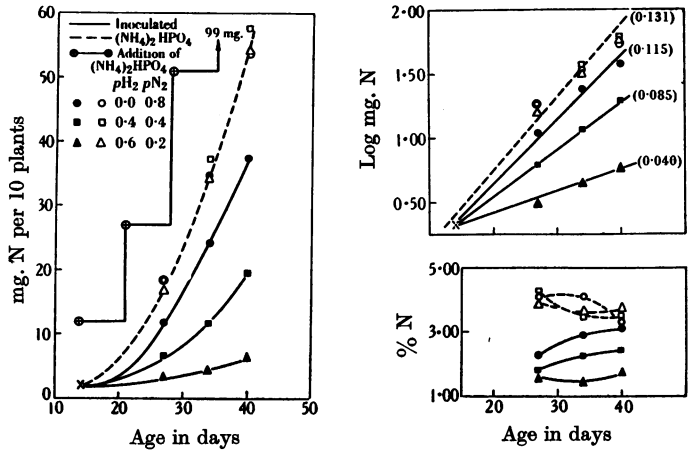

Fig. 2. Effect of pH_2 in atmosphere on assimilation of free and combined N ($(NH_4)_2HPO_4$) by red clover.

(Exp. 2, planted 6 July 1937; all in air until harvest I, 20 July, then changed to indicated atmospheres; harvest II, 2 August; harvest III, 9 August; harvest IV, 15 August.)

The data given in Fig. 2 confirm in every way the conclusions discussed in connexion with Exp. 1. The fit of the experimental points to the straight lines in the semi-log plotting is very satisfactory. These results point to an unmistakable inhibition of the fixation of free N by H_2, an inhibition which is not evident in the assimilation of combined N.

Exps. 3 and 4. These are a part of the series to be discussed in the next section in which the treatment was changed during the course of the tests. Only the semi-logarithmic plots (Fig. 3) are given since these summarize the results of chief interest. Data concerned with the % N were similar to those given in

Figs. 1 and 2. It will be noted that in these experiments the plants receiving combined N were also inoculated. Unless combined N is maintained at a high level, the plants will obtain part of their supply of this element through fixation. In these particular experiments it was desired to keep the rate of development of the plants in the combined N series close to that of the plants dependent entirely on fixation for their N. This was accomplished, as is shown by the g values given in Fig. 3, but the rate of adding NH_4NO_3-N did not entirely prevent fixation of

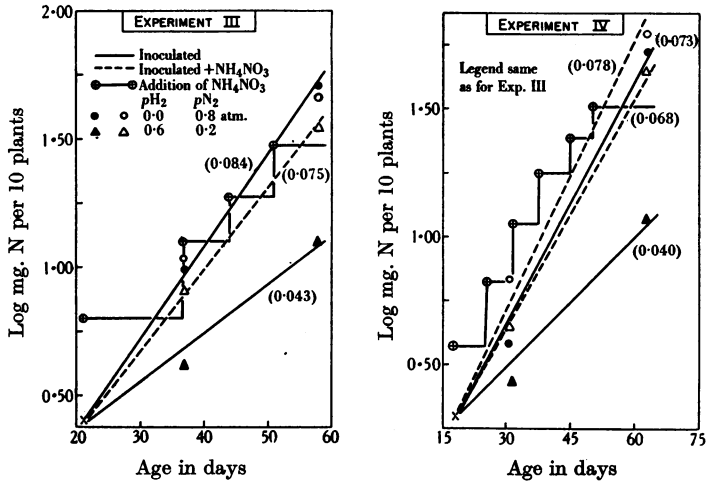

Fig. 3. Effect of H_2 on assimilation of free N_2 and NH_4NO_3 by inoculated red clover plants.

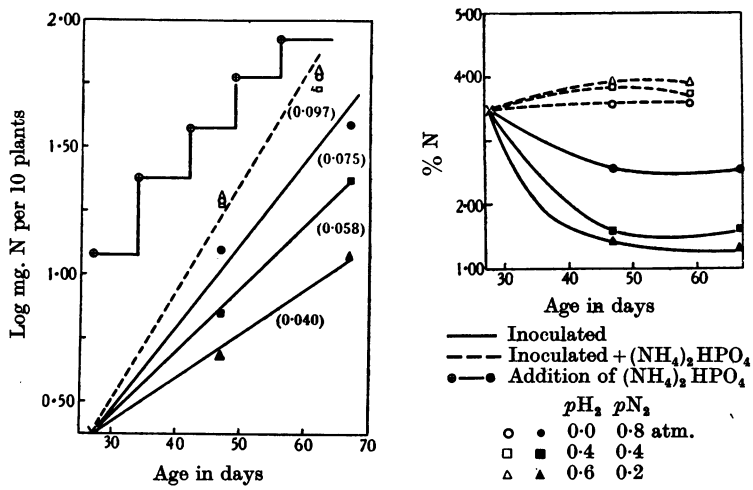

Fig. 4. Effect of presence of H_2 in atmosphere on assimilation of free N_2 and $(NH_4)_2HPO_4$ by red clover.

(Exp. 7, planted 19 August 1937; all kept in air until harvest I, 15 September; then changed to different treatments.)

elemental N by the plants of the combined N series. At harvest, well-developed nodules were found on all the plants. Under such circumstances, it would be expected that the plants of the inoculated plus combined N series which were

grown in the atmosphere containing H_2 would show some evidence of inhibition. As is shown in Fig. 3, such evidence was obtained; the g values for the plants supplied with combined N and kept in an atmosphere with a pH_2 of 0·6 atm. were slightly but significantly lower than those of similar plants kept in air.

Exp. 7. In this experiment the combined N, $(NH_4)_2HPO_4$, was added at such a rate that it was always in excess. As a result fixation of atmospheric N_2 by the plants of the inoculated plus combined N series was entirely suppressed, and the slight inhibition in the development of the plants because of presence of H_2 observed in Exps. 3 and 4 was not obtained (Fig. 4). Plants dependent entirely on fixation for their N showed the "hydrogen effect" both in rate of fixation and in % N in the plants.

The results of these five experiments concerned with the effect of H_2 on the symbiotic N fixation process in red clover consistently support the conclusion that inhibition of N fixation by H_2 persists throughout the growth of the plant and is not merely an initial effect associated with placing the plants in an atmosphere to which they are unaccustomed. The extents of inhibition by a pH_2 of 0·6 atm. as measured by the values of g were as follows: 57, 65, 49, 45 and 47 %. If the total quantity of N fixed is used as the criterion, inhibition reaches 65 to 75% [Wilson & Umbreit, 1937]. The maximum inhibition was observed when the rate of development of the plant was greatest. If environmental conditions are not optimum for growth, fixation is restricted by other factors, e.g. a low supply of carbohydrate, and the extent of inhibition by H_2 is partially masked. Irrespective of the rate of growth of plants in air, the observed g values for plants kept in an atmosphere containing a pH_2 of 0·6 atm. were about 0·040. This means that H_2 was restricting fixation to such an extent that other factors which decrease fixation were relatively ineffective.

The most effective method for decreasing the growth rate of plants kept in air is to reduce the supply of carbohydrate, e.g. by shading. Such treatment would scarcely affect N fixation by plants grown in presence of H_2 since inhibition of fixation by this gas favours the accumulation of carbohydrate, an effect which would compensate for the reduced photosynthesis. The lowest value of g for plants grown in air for which clear-cut inhibition could be readily demonstrated would therefore be in the neighbourhood of 0·050–0·060.

In this connexion it appears that the range of growth rates which may be covered in this type of work is limited. In spite of growing the cultures under widely differing environmental conditions, especially with respect to intensity and duration of light, the g values for inoculated plants kept in the air varied only from 0·073 to 0·115. The g value of 0·115 was observed in an experiment in which the environment was close to ideal for maximum fixation of N. In this same experiment plants supplied with an excess of $(NH_4)_2HPO_4$ assimilated combined N at a rate equivalent to a g value of 0·131. It appears doubtful, then, whether a g value considerably higher than 0·115 could be obtained for fixation of N under the experimental conditions employed.

Change of treatment experiments

The purpose of these experiments was to show that inhibition of N fixation by H_2 was readily reversible and was not associated with any definite stage or rate of growth. In order to demonstrate reversibility of the inhibition, inoculated red clover plants were grown in an atmosphere containing H_2 until inhibition was evident. They were then either changed to an atmosphere free of H_2 or were kept in the H_2-containing atmosphere but supplied with combined N. That the

inhibition was not associated with any particular stage or rate of growth was shown by growing inoculated clover plants in air, then transferring to an atmosphere containing H_2 after fixation had proceeded for various periods of time. Occasionally, in these experiments one of the duplicate cultures of the combined N series would have to be discarded because of chance infection with algae.

Exps. 3 and 4. The data from these experiments are summarized in Table I; briefly, they show that:

Table I. *Effect of change in atmosphere supplied to inoculated red clover plants on assimilation of free N_2 and NH_4NO_3 (Exps. 3 and 4)*

Harvest I to II. Data for harvest II					Harvest II to III. Data for harvest III				
Atmosphere	NH_4NO_3	Total N* mg.	% N	g†	Atmosphere	NH_4NO_3	Total N* mg.	% N	g
Exp. 3:									
Air	–	9·8	2·80	0·084	Hydrogen‡	–	17·9	1·52	0·031
							19·6	1·48	0·035
Air	+	10·9	2·67	0·084	Air	–	47·1	2·95	0·077
							45·6	2·54	0·076
					Hydrogen	–	16·2	1·45	0·028
							22·9	1·52	0·044
Hydrogen	–	4·1	1·60	0·043	Air	–	29·8	2·70	0·087
							26·2	2·67	0·082
					Hydrogen	+	30·0	2·50	0·088
							—	—	—
Hydrogen	+	8·3	1·91	0·075	Air	–	30·7	2·59	0·065
							52·8	3·00	0·090
					Hydrogen	–	18·8	1·55	0·041
							20·5	2·05	0·045
Exp. 4:									
Air	–	4·1	2·58	0·073	Hydrogen	–	19·3	2·07	0·043
		3·6	2·10				19·3	1·69	0·043
Air	+	7·2	3·00	0·078	Air	–	51·8	2·70	0·070
		6·5	2·46				58·8	2·68	0·074
					Hydrogen	–	25·8	1·85	0·047
							25·4	1·80	0·046
Hydrogen	–	2·72	2·08	0·040	Air	–	41·6	3·03	0·081
		2·18	1·58				32·2	2·42	0·073
					Hydrogen	+	22·3	2·63	0·061
							—	—	—
Hydrogen	+	3·8	2·31	0·068	Air	–	60·5	2·81	0·081
		5·1	2·17				66·5	2·87	0·083
					Hydrogen	–	15·7	1·97	0·039
							—	—	—

	Exp. 3	*Exp. 4*
Planted	3 March 1936	2 November 1936
Harvest I	8 April	20 November
II	24 April	3 December
III	15 May	4 January 1937

* Per 10 plants.

† These g values are taken from the lines of Fig. 3; they represent those for the cultures in which the treatments indicated in columns 1 and 2 were unchanged throughout the experiment. Similarly, the g values for harvest III are calculated from the points on these lines at harvest II, rather than the actual experimental points.

‡ Hydrogen = pH_2, 0·6 atm.; pN_2, 0·2 atm.; pO_2, 0·2 atm.

All cultures were inoculated and kept in air until harvest I, then changed to treatments indicated in columns 1 and 2, until harvest II, and finally changed to treatments indicated in columns 6 and 7.

(1) If inoculated clover plants are grown in air and then transferred to an atmosphere containing 0·6 atm. H_2, the rate of N fixation decreases from the high value characteristic of plants kept in air to that of plants which have been continuously maintained in an atmosphere of this pH_2.

(2) If clover plants which have been both inoculated and supplied with combined N are grown in air and after a period have their fixed N supply withdrawn, assimilation of free N proceeds at a rate characteristic of plants fixing N in air during the entire experiment. The slight decrease in the observed value of g probably arises from a lag in the development of the nodules occasioned by the presence of combined N in the early stages of development. If the plants are transferred to an atmosphere containing a pH_2 of 0·6 atm. when the supply of fixed N is withdrawn, fixation of elemental N is inhibited so that the g value with such plants drops to one equal to, or perhaps slightly higher than, that characteristic of inoculated plants given no combined N and kept in the H_2-containing atmosphere throughout the experiment. The slightly higher values occasionally encountered are most likely to be ascribed to residual combined N remaining in the substrate when the transfer is made.

(3) If inoculated plants which have been grown in an atmosphere containing H_2 on a substrate free of combined N are transferred to air, the rate of fixation increases from a low value to one that is typical of plants continuously kept in air. Moreover, if the plants are kept in the H_2-containing atmosphere but supplied with combined N, there is an immediate increase in the rate of assimilation to a value characteristic of plants supplied with combined N throughout the experiment.

(4) If inoculated clover plants are supplied with combined N and grown in an atmosphere with a pH_2 of 0·6 atm., the rate of N assimilation is only slightly less than that of similar plants grown in air (see discussion of Exps. 3 and 4 in the preceding section). If the supply of combined N is withdrawn, and the plants are kept in the same atmosphere, the rate of assimilation of free N assumes a value characteristic of inoculated plants grown in the presence of this pH_2. If, however, at the time of withdrawal of the combined N the plants are transferred to air, assimilation of free N proceeds at a rate typical of inoculated plants grown in air.

In all these various responses corresponding changes in the % N in the plants occurred coincidently with the changes in the rate of assimilation of N. It is clear from these results that the inhibition of N fixation by molecular H_2 is independent of the previous growth history of the plant and that the effect is readily reversible, that is, transfer from a H_2-containing atmosphere to air results in an increase in the rate of fixation and *vice versa*.

Exp. 5. In this experiment all plants were inoculated and kept in air until N fixation had begun, when all were placed in an atmosphere with a pH_2 of 0·6 atm. When inhibition of fixation had proceeded to the stage where the plants turn yellow because of excessive carbohydrate, four series of different treatments were formed. Plants of *Series 1* were transferred to air; those of *Series 2* were kept in the atmosphere containing H_2, but an excess of combined N as $(NH_4)_2HPO_4$ was added; plants of *Series 3* were treated as those of *Series 2* except that the combined N was supplied as $Ca(NO_3)_2$; plants of *Series 4* were kept in the H_2 atmosphere with no treatment. The essential data from this experiment which are shown in Fig. 5 provide additional evidence that the action of H_2 in the atmosphere is definitely concerned with the N fixation process and not with the general growth of the plant. If plants are returned to air after inhibition of fixation by H_2, the rate of N fixation soon reaches a value characteristic of fixation in air. This demonstrates that the effect of the H_2 is reversible. It is unnecessary, however, to return the plants to air in order to bring about normal development as this also can be accomplished by supplying combined N to the plants which are maintained in the atmosphere containing H_2. Coincidently with the increase in rate of fixation (or assimilation of combined N) after change in treatment there occurs an increase in the percentage N in the plants.

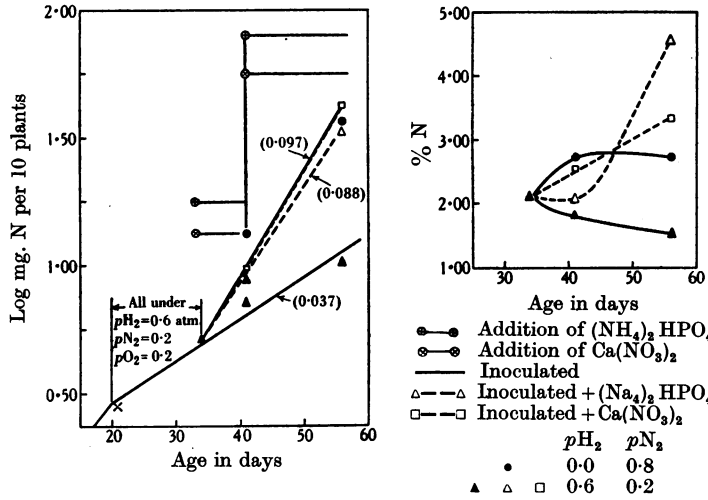

Fig. 5. Effect of change in treatment on assimilation of free and combined N by red clover.

(Exp. 5, planted 2 February 1937; all kept in air until harvest I, 19 March, then placed in H_2-containing atmosphere; treatments changed as indicated at harvest II, 2 April; harvest III, 19 April; harvest IV, 24 April.)

Table II. *Effect of source of N on inoculated red clover plants in presence and absence of H_2 (Exp. 6)*

Atmosphere	Source of N	Total N mg.	% N	*g*	Addition of combined N
Exp. 6:					
Air	N_2	30·9	3·12	0·152	2 Aug. —24·2 mg. NH_4^+–N
		30·9	2·96	0·152	
	$Ca(NO_3)_2$	16·5	3·62	0·108*	23·9 mg. NO_3^-–N
		27·6	3·61	0·144	
	$(NH_4)_2HPO_4$	42·8	3·44	0·176	10 Aug.—24·2 mg. NH_4^+–N
		32·9	3·81	0·157	
					23·9 mg. NO_3^-–N
pH_2=0·6 atm.	N_2	5·34	1·73	0·028	
pN_2=0·2 atm.		7·18	1·84	0·049	
pO_2=0·2 atm.		4·91	1·60	0·022	
	$Ca(NO_3)_2$	8·06	2·18	0·057*	
		33·8	3·51	0·157	
	$(NH_4)_2HPO_4$	26·0	3·45	0·140	
		28·5	3·57	0·147	

* See text for discussion of these values.

All cultures were inoculated and kept in air until harvest I; all changed to an atmosphere of pH_2=0·6, pN_2=0·2 and pO_2=0·2 atm. until harvest II; then to treatments indicated in columns 1 and 2 of the table.

Planted	6 July 1937
Harvest I	20 July (2·12)
II	2 August (3·63)
III	16 August

Figures in parentheses refer to nitrogen content of 10 plants at indicated harvest.

This experiment was made simultaneously with Exp. 2 (Fig. 2). The *g* values for cultures kept in air throughout experiment were:

Inoculated, 0·115;
Inoculated plus $(NH_4)_2HPO_4$, 0·131.

Those for cultures kept in pH_2 0·6 atm. were:

Inoculated, 0·040;
Inoculated plus $(NH_4)_2HPO_4$, 0·131.

Exp. 6. This is of chief interest as it illustrates a type of variation in response to change in treatment which is occasionally encountered, viz. delay in the recovery of plants on addition of combined N after these have been kept under a rather high pH_2 for some time. The experiment, the general plan of which was identical with that of Exp. 5, was conducted during the summer under conditions which favoured a high rate of photosynthesis. As a result the plants which had been kept in the H_2-containing atmosphere developed an excessive carbohydrate-nitrogen balance as evidenced by yellow leaves and red stems. Inoculated plants transferred to air responded immediately to the change in treatment, and in a few days the colour of the plants was changing to green. N fixation was evidently stimulated by the excessive carbohydrate [Wilson, 1935] since the value for g, 0·152, was the highest obtained in these experiments for assimilation of free N_2 (see Table II). The response of the plants given an excess of combined N (which would effectually prevent fixation of the element) was somewhat erratic. Uptake of the combined forms started immediately in the majority of the plants, but two cultures which were furnished $Ca(NO_3)_2$ did not begin assimilation for several days as judged by appearance of green colour in leaves and stems. After 7–10 days, assimilation appeared to take place normally, but the initial lag caused the plants to contain less N at harvest than did the others so that the calculated values of g for these two cultures were definitely lower.

Exp. 7. In this experiment further evidence of the occurrence of a lag in uptake of combined N by red clover plants previously kept in an atmosphere

Table III. *Effect of change in the atmosphere on assimilation of free and combined N (Exp. 7)*

Harvest I to II				Harvest II to IV		Data of harvest III			Data of harvest IV		
Treatment	Total N mg.	% N	g*	Atmosphere	Source of N	Total N mg.	% N	g	Total N mg.	% N	g
Exp. 7:											
Air	13·1	2·64	0·075	Hydrogen†	N_2	21·4	1·90	0·047	—	—	—
						18·7	1·75	0·038	24·3	1·76	0·029
pH_2=0·6 atm.	4·63	1·46	0·040	Air	N_2	24·0	3·03	0·103	—	—	—
pN_2=0·2 atm.						22·0	3·14	0·097	38·8	2·75	0·075
pO_2=0·2 atm.				Air	NH_4	—	—	—	62·9	3·55	0·105
						30·3	3·93	0·118	44·0	3·76	0·054
				Air	NO_3	19·6	2·94	0·089	36·2	3·00	0·051
						31·2	3·43	0·120	40·0	3·49	0·065
				Hydrogen	NH_4	24·2	3·37	0·103	56·8	3·50	0·131
						20·8	2·79	0·093	42·4	3·52	0·091
				Hydrogen	NO_3	34·6	3·94	0·127	—	—	—
						25·2	3·29	0·106	46·8	3·41	0·064

* These values of g are for cultures kept in atmosphere indicated in column 1 throughout experiment (see Fig. 4).

† Hydrogen=pH_2, 0·6 atm.; pN_2, 0·2 atm.; pO_2, 0·2 atm.

All cultures planted and inoculated—19 August 1937.

Harvest I Placed under atmospheres given in column 1—15 September.
II Changed to treatment given in columns 5 and 6—5 October.
III 20 October.
IV 27 October.

Addition of combined N:

	$Ca(NO_3)_2$	$(NH_4)_2HPO_4$
6 October	24·2 mg.	24·2 mg.
12	24·2 mg.	24·2 mg.
20	24·2 mg.	24·2 mg.

containing H_2 was obtained. Two harvests were made following the change of treatment, but since the rates of fixation were not always constant, values of g were calculated for each harvest. The results, summarized in Table III, confirm those of the previous experiments. If inoculated red clover plants which have been grown in air until N fixation is actively under way are transferred to an atmosphere containing a pH_2 of 0·6 atm., there is an immediate decrease in the rate of fixation. On the other hand, if the plants are first kept in the H_2-containing atmosphere until a carbohydrate excess accumulates, and then placed in air, decided acceleration in the rate of N fixation occurs. But if such plants are furnished with sufficient combined N to stop fixation of the free element, uptake of the combined forms may be delayed for several days. After a lag period of 7 to 10 days assimilation of combined N begins, and the plants develop rapidly. They do not, however, succeed in overtaking those cultures which begin the assimilation of combined N immediately after its addition.

The cause of this unexpected lag in the uptake of combined N in some of the plant cultures is unknown, but the following characteristics of its occurrence should be noted.

(1) The lag is observed only in plants which have an excess of carbohydrate as a result of being placed in an atmosphere which restricts fixation of elemental N_2. In some respects its occurrence is the reverse of that previously noted by Fred *et al.* [1938] who found that soy beans high in carbohydrates began assimilation of free N_2 only after addition of small quantities of combined forms of this element.

(2) It is noteworthy that fixation of elemental N_2 by these clover plants was unaffected by the previous growth in the H_2-containing atmosphere. This difference in the assimilation of free and combined N might be used as support for the view of Virtanen & v. Hausen [1931] that the element is a superior source of N for nutrition of the clover plant.

(3) The lag period is not a typical response since, with the majority of the cultures, uptake of combined N occurred almost immediately after its addition, and certainly as soon as fixation of the free element could be detected in the inoculated cultures transferred to air.

(4) The delay in assimilation of combined N does not appear to be associated with the presence of H_2 since some of the plants transferred to air likewise exhibited the lag period.

Discussion

The experiments reported in this paper together with those previously discussed [Wilson & Umbreit, 1937] provide four types of evidence for the view that H_2 is a specific inhibitor of N fixation by the symbiotic system in red clover. These are:

1. The total quantity of N fixed by inoculated red clover plants is linearly dependent on the pH_2 in the atmosphere, whereas the assimilation of combined N is independent of the pH_2 within experimental error.

2. Clover plants which have been grown in atmospheres of different partial pressures of H_2 show significant differences in the total quantity of elemental N_2 fixed, but similar plants supplied with combined N do not assimilate significantly different quantities of the combined forms.

3. The rates of assimilation of both free and combined N are essentially logarithmic during the period of growth under study which allows calculation of the unimolecular constant of N uptake—the so-called g value. Values of g are significantly different for plants grown in atmospheres of differing pH_2 only if the plants must use free N_2 for their source of this element.

4. Inhibition by H_2 is obtained at different stages of growth and is reversible. For example, inoculated clover plants transferred from an atmosphere containing H_2 to air immediately show an increase in the rate of fixation. Moreover, if the

plants are not transferred but supplied instead with combined forms of the element, uptake of the combined N and development of plants proceed at rates which are strictly comparable with those of similar plants kept in air.

Seventeen experiments have been made during 5 years with consequent fairly large variations in the environmental factors which will influence assimilation of either free or combined N, e.g. intensity and duration of light, temperature etc. The results of all the experiments consistently point to the view that the action of H_2 on the development of inoculated red clover plants is specifically associated with N fixation. All attempts to detect a significant effect on the general development of the plant apart from the fixation of free N_2 or to correlate the action with any particular stage or rate of growth were unsuccessful. On the basis of these data, it is therefore concluded that H_2 is a specific inhibitor for the symbiotic N-fixing system in red clover.

Summary

Additional evidence has been furnished which confirms the conclusion reached from results of previous work: molecular H_2 is a specific inhibitor for the symbiotic N fixation process in inoculated red clover plants.

The assimilation of both free and combined N by red clover under the conditions of the reported experiments is sufficiently close to logarithmic to allow calculation of the unimolecular constants of N assimilation, the g values. These constants are particularly useful for detection of stimulating or inhibiting effects of a substance on the rate of a given reaction. Values of g for assimilation of free N_2 were significantly decreased through addition of H_2 to the atmosphere, but those for assimilation of combined N were independent of the presence of H_2 in the atmosphere.

That the inhibitory action of H_2 is not associated with any particular stage or rate of growth was demonstrated by transferring plants to an atmosphere containing H_2 after growing in air for various periods of time. It was also shown that the action was reversible since plants kept in the presence of H_2 immediately increased their rate of N assimilation if transferred to air or if combined N was supplied to them.

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