[CONTRIBUTION FROM DEPARTMENT OF AGRICULTURAL BACTERIOLOGY, UNIVERSITY OF WISCONSIN]

Mechanism of Biological Nitrogen Fixation. VIII. Carbon Monoxide as an Inhibitor for Nitrogen Fixation by Red Clover[1]

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Knowledge of the mechanism of biological nitrogen fixation has been definitely advanced recently through applying physical-chemical techniques to investigation of the responsible enzyme system.[2,3] Discovery of specific inhibitors, *e. g.*, hydrogen, for the fixation reaction has proved especially valuable in furthering our understanding of the properties of this system. Some years ago we[4] surveyed the effects of the commonly used enzyme inhibitors on the nitrogen fixation reaction in inoculated red clover plants. Apart from hydrogen, and with the exception of carbon monoxide, no specific activity, *i. e.*, differential effect with plants using free and combined nitrogen, was observed. Because very small concentrations of carbon monoxide were found to inhibit fixation, it was believed at first that this gas occurring as an impurity in tank hydrogen might cause the rather unexpected inhibition observed with the latter. Later investigations proved this explanation to be untrue.[4] Further studies on the nature of the hydrogen inhibition necessitated postponing more detailed investigation of the carbon monoxide effect until recently. The results of these later experiments are reported in this paper.

Experimental

Methods.—The methods for growing the plants have already been described by Wilson.[5] Briefly, 20 clover plants were grown on a sand substrate in 9-liter Pyrex bottles provided with facilities for changing the atmosphere furnished the plants. A solution containing all necessary plant nutrients except combined nitrogen was added to the sand. Part of the plant cultures were inoculated with an efficient strain of *Rhizobium trifolii;* the remainder were uninoculated but supplied periodically with ammonium nitrate. Carbon monoxide, prepared from formic acid and concentrated sulfuric acid, was washed through a chain consisting of alkaline permanganate, distilled water and a cotton plug. Analysis of carbon monoxide in the atmospheres was made by a modification of the method described by Christman, Block and Schultz.[6]

Total Nitrogen Experiments.—Several exploratory trials established the pressure range over which carbon monoxide acts specifically on the nitrogen fixation reaction. In these experiments the total nitrogen in the plants at the start of the treatments and after about one month was determined. Typical results from three such experiments are given in Table I; the values represent the averages of duplicate or triplicate cultures. They show that definite inhibition of nitrogen fixation by carbon monoxide can usually be observed when the concentration of this gas reaches 0.01% and that practically complete inhibition is noted at 0.05–0.1%. In this range, however, the uptake of combined nitrogen is hardly affected, and even when extended to 0.3%, as in experiment 3, the inhibition, if any, is small. Under the experimental conditions used, *i. e.*, a closed system, we have always observed more variable results with plants supplied combined nitrogen in comparison with those using the atmospheric form.

TABLE I

INFLUENCE OF CARBON MONOXIDE ON UPTAKE OF FREE AND COMBINED NITROGEN BY RED CLOVER (TOTAL NITROGEN EXPERIMENTS)

% CO in atm.	Expt. I	Expt. II	Expt. III
	Inoculated Series		
0.00[a]	44.5[b]	25.0	28.7
.005	39.6	26.4	..[c]
.01	35.4	24.0	18.0
.02	23.7	14.4	7.5
.03	14.7	..	..
.05	6.9	3.8	4.1
.1	..	7.5	3.0
Nitrogen at first harvest[d]		3.1	3.2
	Ammonium Nitrate Series		
0.00[a]	30.3	26.1	26.1
.005	29.8	18.4	..
.01	28.4	24.1	25.2
.02	32.0	24.5	26.9
.03	37.7	..	..
.05	31.3	24.8	18.9
.1	..	21.0	19.8
.2	..	..	25.6
.3	..	..	19.1
Nitrogen at first harvest[d]		3.7	4.0

[a] Air control. [b] Values in all tables refer to mg. N per 10 plants. [c] Blank indicates that particular concentration of CO was not used. [d] First harvest made at time plants placed in CO atmospheres.

Expt.	Planted	CO started	Harvested	Total NH_4NO_3 added
I	6/11/40	7/17/40	8/20/40	40.5[b]
II	8/30/40	10/2/40	11/1/40	26.3
III	11/16/40	12/20/40	1/24/41	26.7

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(2) Burk and Burris, *Ann. Rev. Biochem.*, **10**, 587 (1941).

(3) Wilson, "Biochemistry of Symbiotic Nitrogen Fixation," University of Wisconsin Press, Madison, 1940.

(4) Wilson, *Ergeb. Enzymforsch.*, **8**, 23 (1939).

(5) Wilson, THIS JOURNAL, **58**, 1256 (1936).

(6) Christman, Block and Schultz, *Ind. Eng. Chem., Anal, Ed.*, **9**, 153 (1937).

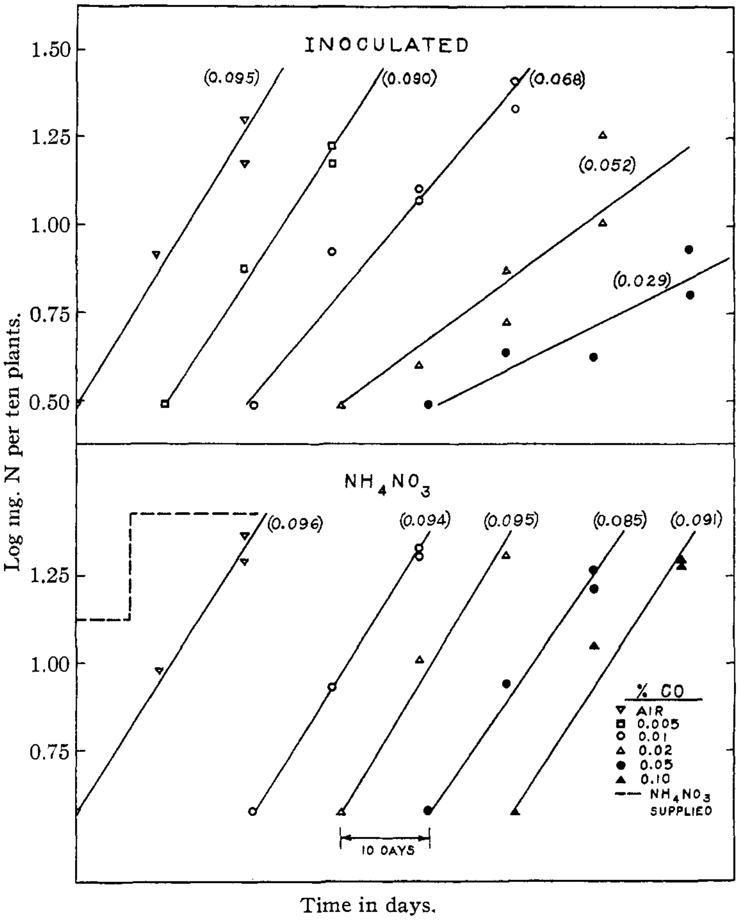

Fig. 1.—Effect of carbon monoxide on uptake of free and combined nitrogen by red clover. (In both Figs. 1 and 2 a sliding scale is used for the abscissa so that the individual lines are distinct; zero for each line is represented by the initial point. The figures in parentheses give the k values corresponding to each line. Addition of ammonium nitrate-N, shown by dotted line corresponding to air control was 13.2 mg. N per 10 plants at start; 13.2 mg. supplied after seven days.)

Rate Experiments.—Following these preliminary trials, more precise studies were made in which periodic harvests of the plants allowed the rates of fixation to be determined, a procedure which has definite experimental advantages for this type of study.[3,7] Results of two such experiments are shown in Figs. 1 and 2. When the log mg. N per 10 plants is plotted against time in days, a reasonably close linear relationship is obtained. The slope of this line, estimated by the usual statistical procedure, multiplied by 2.303 gives the specific monomolecular rate constant of fixation, k. The statistical treatment also provides a method for testing the significance of an observed difference in the slopes of the lines and therefore in the values of k. In agreement with the previous results, 0.01% carbon monoxide in the atmosphere definitely inhibited nitrogen fixation. The inhibition increased with the pCO until the latter reached about 0.05%. At this concentration the plants fixed very little nitrogen, turned yellow, and practically ceased to grow. If, however, ammonium nitrate was supplied replicate plants, the k's for uptake of nitrogen were the same within experimental error independent of carbon monoxide concentration.

Figure 2 presents results of an experiment which consisted entirely of plants supplied combined nitrogen, and in which the concentration of carbon monoxide ranged from 0.01 to 0.5%. Statistical analysis indicated that a significant difference in the k's must be at least 0.021; obviously the observed k values are identical within experimental error. The data in Table II from another rate experiment show a small but significant decrease in the values of k for uptake of combined nitrogen within the range 0.5–2.0%. Even at the relatively high level of 2% carbon monoxide, however, the decrease in the k value for assimilation of ammonium nitrate was only 25%; in contrast, at the much lower concentration of 0.05% carbon monoxide, inhibition of free nitrogen fixation was 68% as judged by the decrease in the k value.

Discussion

Carbon monoxide and molecular nitrogen have the same molecular weight, same number of valence electrons, and possess many physical properties which are remarkably similar. Because of these similarities, inhibition of the uptake of atmospheric nitrogen by carbon monoxide might be regarded as a natural consequence of the competition of these two gases for adsorption on the surface of the responsible enzyme. One experiment only has been completed in which the nature of the competition was studied. As shown in Table III, inhibition based on either total nitro-

TABLE II

EFFECT OF CARBON MONOXIDE ON RATE OF UPTAKE OF FREE AND COMBINED NITROGEN BY RED CLOVER

% CO in atm.	Final total N content N$_2$ series	Final total N content NH$_4$NO$_3$ series	k values[a] N$_2$ series	k values[a] NH$_4$NO$_3$ series
0.00	27.60	37.7	0.083	0.093
.01	21.30	46.3	.076	.094
.02	10.33	40.6	.056	.095
.05	4.23	34.6	.027	.094
.1	3.48	35.0	.026	.091
.5		25.7		.081
1.0		28.0		.082
2.0		18.3		.069

[a] Based on three harvests (5 points). Nitrogen in plants when placed in carbon monoxide atmospheres: N$_2$ series, 1.95; NH$_4$NO$_3$ series, 1.86 mg.

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(7) Burk, *Ergeb. Enzymforsch.*, **3**, 23 (1934).

gen fixed or rate of fixation (calculated from initial and final nitrogen content) apparently depends only on concentration of carbon monoxide and is independent of the pN_2. Analysis of the results by the method of Lineweaver and Burk[8] confirmed this conclusion. When $1/k$ was plotted against $1/pN_2$ for different concentrations of carbon monoxide, the following lines were obtained

% CO	Line of regression	t^a	$t_{0.05}$
0.00	$1/k = 13.18 + 0.412\ 1/pN_2$	1.06	2.77
.01	$1/k = 15.98 + 0.075\ 1/pN_2$	0.10	2.57
.02	$1/k = 27.07 + 1.482\ 1/pN_2$	1.00	2.36
.03	$1/k = 38.00 - 0.039\ 1/pN_2$	0.10	2.45

[a] t value obtained when slope was tested against zero; must exceed 5% point ($t_{0.05}$) to be significant. For competitive inhibition slopes of lines of carbon monoxide must be significantly greater than zero.

Only in the 0.02% carbon monoxide series was a slope obtained which appeared to differ significantly from zero. The experimental error was considerably larger than usual in this case, however, and the value of t indicated that on the basis of this single trial, there was no regression on $1/pN_2$. If the carbon monoxide does inhibit through chemical combination with some component of the nitrogen fixing enzyme—for example, a heavy metal—the effect is not permanent but appears to be readily reversible as is shown by the data in Table IV.

TABLE III

NATURE OF INHIBITION BY CARBON MONOXIDE OF NITROGEN FIXATION IN RED CLOVER

% CO	pN_2 in atm. 0.8	0.4	0.2
	Mg. N fixed		
0.00	27.87	22.42	22.60
.01	18.25	19.86	17.52
.02	7.66	6.50	5.30
.03	4.47	5.67	4.97
	k values		
.00	0.0745	0.0685	0.0674
.01	.0630	.0634	.0617
.02	.0379	.0333	.0296
.03	.0247	.0307	.0271

Nitrogen in plants when placed in carbon monoxide atmospheres, 1.77 mg.

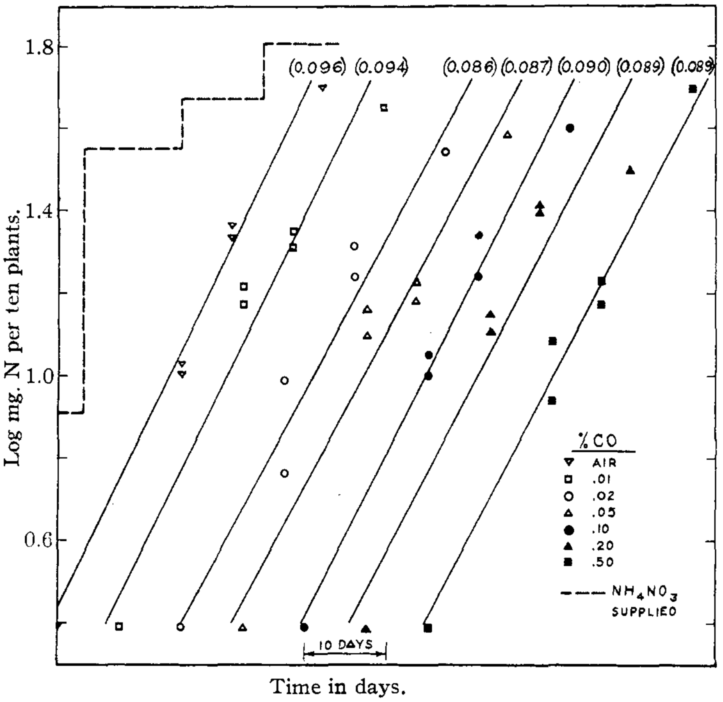

Fig. 2.—Effect of carbon monoxide on uptake of ammonium nitrate by red clover. (In both figures the addition of ammonium nitrate to the combined nitrogen series, which was identical for all treatments, is shown only for the air control.)

Although these results point to a *specific* influence of carbon monoxide on nitrogen fixation in the range 0.01–0.1%, this conclusion is not so clear-cut as in the case of inhibition by hydrogen.[3] The inhibition of plants given combined nitrogen at higher concentrations of carbon monoxide points to a *non-specific* effect on the growth of the plant independent of the source of nitrogen. The fact that the non-specific activity requires considerably higher concentrations for quantitatively inferior inhibition suggests that this latter effect may be an additional one distinct from the specific inhibition of free nitrogen assimilation. The non-specific effect does not appear to arise from inhibition of respiration since the concentration involved is much less than that necessary for inhibition of the *Atmungsferment;* it is more of the order of magnitude noted for competition between carbon monoxide and oxygen for hemoglobin. This difference in the response of plants supplied combined nitrogen, in comparison with those fixing nitrogen apparently is not related to differences in the rates of growth, since the experimentally observed values of k for the air controls do not differ greatly. Neither is it due to assimilation of car-

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(8) Lineweaver and Burk, THIS JOURNAL, **56**, 225 (1934).

TABLE IV

REVERSIBLE NATURE OF CARBON MONOXIDE INHIBITION

% CO in atm.	Controls[a]	Treated[b]
0.00	32.40	
.01	17.38	22.96
.02	7.86	18.20
.05	4.51	13.68
.1	4.97	10.57
.2	4.54	11.84

[a] In CO atmospheres from 12/14/40 to 1/11/41. [b] In CO atmospheres from 12/14/40 to 12/28/40; in air from 12/28/40 to 1/11/41.

bon monoxide in the presence of ammonium nitrate—for example, by chance contaminating microörganisms—since estimation of this gas in the atmospheres during the course of the experiments showed no detectable change.

To settle these points definitely would require many carefully controlled experiments in which the external environment, including source of nitrogen, would be varied. Such investigations would be laborious, time-consuming and possibly indecisive, since accurate control of all variables is very difficult in long-time experiments. Another method, however, is available which offers less experimental limitation. Wyss and Wilson[9] have recently demonstrated that the symbiotic nitrogen fixation system in red clover is probably identical with that in *Azotobacter*. Consequently, carbon monoxide should likewise inhibit fixation by this organism. Preliminary experiments have shown this to be true and, although the actual ranges differ, the results are entirely analogous: at low concentrations of carbon monoxide, uptake of free nitrogen is inhibited—at higher levels, uptake of combined nitrogen is inhibited as well. Since *Azotobacter* can be used in short-time trials (two to five hours in the Warburg respirometer), the use of this organism affords definite advantages for more precise and detailed investigation of the mechanism of carbon monoxide inhibition. Further study of this reaction is accordingly being made with *Azotobacter*.

Summary

Evidence from experiments in which total nitrogen fixed was estimated, as well as others in which rate of fixation was measured, shows that uptake of free nitrogen by inoculated red clover plants is inhibited by as little as 0.01% carbon monoxide. The fixation process is practically stopped by 0.05% carbon monoxide. In this range of *p*CO, no effect on assimilation of ammonium nitrate by uninoculated plants is observed, but at higher concentrations definite inhibition is detectable. The inhibition of nitrogen fixation appears to be reversible and non-competitive.

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[9] Wyss and Wilson, *Proc. Natl. Acad. Sci. U. S.*, **27**, 162 (1941).

[CONTRIBUTION FROM THE CHEMISTRY LABORATORY, UNIVERSITY OF MISSOURI]

The Action of Aluminum Chloride on Aromatic Hydrocarbons. III. The Polyethylbenzenes and Tetramethylbenzenes

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Previous work on the action of aluminum chloride on aromatic hydrocarbons in this Laboratory has been with trialkylbenzenes of the type 1,3-dimethyl-4-R-benzenes where R = C_3H_7 and C_4H_9.[2,3] We have now completed a similar study of the triethylbenzenes, the tetraethylbenzenes and the tetramethylbenzenes.

The *sym*- and *unsym*-triethylbenzenes were each converted into a mixture of these two hydrocarbons along with some diethylbenzene and tetraethylbenzene by the action of aluminum chloride at 70–75°. In the case of the 1,3,5-triethylbenzene, the ratio of the two isomers as separated from the trialkyl fraction was approximately 1 part 1,2,4-isomer to 2.5 parts 1,3,5-isomer, while from 1,2,4-triethylbenzene the ratio was 1:1.5. Losses of hydrocarbon during sulfonation and subsequent hydrolysis of the triethylbenzene sulfonic acids were about the same for each of the two hydrocarbons so these ratios should be representative of the composition of the total triethylbenzenes present in the mixture. This is the first time that it has been possible to obtain a 1,2,4-hydrocarbon from a 1,3,5-hydrocarbon.

The *sym*- and *unsym*-tetraethylbenzenes were each partially converted into the other, as were

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[1] Abstract of a dissertation presented by Francis Wadsworth in partial fulfillment of the requirements for the Degree of Master of Arts at the University of Missouri.

[2] Nightingale and Carton, THIS JOURNAL, **62**, 280 (1940).

[3] Nightingale and Smith, *ibid.*, **61**, 101 (1939).