[CONTRIBUTION FROM THE DEPARTMENTS OF AGRICULTURAL BACTERIOLOGY AND AGRICULTURAL CHEMISTRY, UNIVERSITY OF WISCONSIN]

# Mechanism of Symbiotic Nitrogen Fixation. I. The Influence of $pN_2$[1]

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Research on fixation of elemental nitrogen through association of leguminous plant and bacteria (*Rhizobia sp.*) has been conducted for almost a century, but the chemical mechanism of the process has remained practically unattacked. The scanty nature of our knowledge of this aspect of the problem arises mainly from lack of suitable techniques to carry on the research; the isolation of intermediates, which has been so successful in elucidating the mechanism of a fermentation reaction, is not readily applied because of difficulties in separating from the plant cells products which may be definitely ascribed to the fixation process.

Application of physical chemistry techniques to the problem hitherto has not been attempted.[2] Even without definite knowledge of the exact nature of the key-reactions, many of their properties may be ascertained by determining the effect of controlled changes in the physical and chemical environment, *e. g.*, temperature, *p*H, oxidation–reduction potentials, and pressure of gases (nitrogen, oxygen, carbon dioxide). In this paper are discussed the effects of changes in the $pN_2$ of the atmosphere supplied inoculated clover plants on the fixation reaction.

## Experimental and Results

**Technique.**—Red clover (*Trifolium pratense*) was used as the host plant and inoculated with an efficient strain of the proper organism, *Rhizobium trifolii*. Twenty plants were grown in a 10-liter Pyrex pressure bottle on a nitrogen-poor sand substrate to which was added the required plant nutrients. An atmosphere of any desired composition was maintained in these bottles by evacuation followed by addition of the required gases. Cylinder gases, passed through a purifying chain of sodium hydroxide, sulfuric acid, alkaline potassium permanganate, distilled water, and finally a sterile cotton filter were used in all the experiments. The atmospheres were renewed twice a week; the $pCO_2$ was maintained inside the bottles at 0.1 to 0.5% by the method described by Smyth.[3] In a few experiments 64-oz. (2-liter) flint glass bottles were used as the plant container, but these were not so satisfactory because of difficulty in maintaining uniform $pCO_2$ and of the restriction on the growth of the plant. The experiments were conducted in a greenhouse equipped with artificial lighting and other means for controlling light and temperature.[4] At the start of each experiment the plants were supplied with air containing 0.1% carbon dioxide until fixation was well under way and the period of nitrogen hunger was passed. They were then transferred to the various atmospheres and allowed to grow for fifteen to fifty-five days before harvest. No combined forms of nitrogen were furnished the inoculated plants, but a sufficient quantity of all other plant nutrients was available; hence the limiting factor for growth was primarily rate of fixation of free nitrogen. Plants given combined nitrogen were supplied with 3 to 5 mg. of $NH_4NO_3$–N per bottle at the start which enabled them to be at the same stage of development as the inoculated plants at the time atmospheres were first changed. Five to 10 mg. of $NH_4NO_3$–N was then added weekly to all cultures of the combined nitrogen series depending on the rate of growth maintained by the plants; this addition of $NH_4NO_3$–N was uniform to all plants in the combined nitrogen series in a given experiment, *i. e.*, each culture received the same quantity at each time of addition.

In the first experiments it was found that replicates of the same treatment might vary 10 to 20% in total nitrogen per 10 plants at time of harvest. In an effort to reduce the variation, plant cultures *in a given experiment* were selected for uniformity at time they were placed under the different atmospheres and various technical devices employed, *e. g.*, rotation of cultures on the benches in the green house, in an effort to eliminate the non-uniformity of environmental factors. In spite of these precautions variations of 5 to 10% frequently were observed in duplicates. Since plant growth tends to be logarithmic in the early stages of development, initial small differences may be increased considerably unless the experiments are extremely short-time in nature. The best solution of this technical difficulty appears to be to draw conclusions only after numerous replications of the experiments have been made under conditions which will vary the *rate* and *extent of growth* together with a statistical analysis of the data. In this report data are considered from a total of 20 experiments covering a time period of two years. The experiments were made during all seasons with consequent differences in (1) rate of growth (average daily gain per 10 inoculated plants has varied from 0.2 to 1.0 mg.); (2) extent of growth (from 5 to 35 mg. total nitrogen per 10 plants); (3) length of experiment (from 15 to 57 days); and (4) initial size of plant (initial nitrogen content from 1.05 to 3.50 mg.).

Preliminary experiments were made as follows: one series of clover plants was grown under a vacuum of 0.5 atm., a second at atmospheric pres-

(1) Herman Frasch Foundation in Agricultural Chemistry, Paper No. 119.

(2) Burk and collaborators have been very successful in studying the mechanism of fixation by *Azotobacter* through the physical chemical approach; [*Ergebnisse Enzymforsch*, **3**, 23 (1934)]. The Warburg technique used by Burk would not be applicable to the symbiotic process so long as fixation must be accomplished with the association of bacteria and plants.

(3) Smyth, *Science*, **80**, 294 (1934).

(4) Wilson and Georgi, *Bot. Gaz.*, **94**, 346 (1932).

sure and a third at a pressure of 1.8 atm. maintained by added nitrogen gas. In this manner plants developed under $pN_2$ of 0.39, 0.78 and 1.56 atmospheres; under these conditions it was found that the fixation of nitrogen is independent of the $pN_2$ used as is indicated by the data in Table I, experiments I and II.

These observations were confirmed and extended by experiments in which the $pN_2$ was varied from 0.06 to 0.80 atm., the $pO_2$ being kept constant in a given experiment, and no inert gas being added, *i. e.*, the plants were grown under dif-

TABLE I

EFFECT OF $pN_2$ ON NITROGEN FIXATION OF RED CLOVER IN ABSENCE OF INERT GAS

| $pN_2$, atm. | Total pressure, atm. | Dry weight, mg. | Total N, mg. | % N |
|---|---|---|---|---|
| | | Expt. I | | |
| 0.39 | 0.50 | 760 | 21.42 | 2.82 |
| .78 | 1.00 | 877 | 24.10 | 2.75 |
| 1.56 | 1.76 | 805 | 21.50 | 2.67 |
| | | Expt. II | | |
| 0.39 | 0.50 | 1045 | 26.75 | 2.56 |
| .78 | 1.00 | 1032 | 26.40 | 2.56 |
| 1.56 | 1.76 | 1174 | 29.50 | 2.51 |
| | | Expt. III | | |
| 0.75 | 1.00 | 366 | 7.65 | 2.09 |
| .20 | 0.45 | 323 | 6.55 | 2.03 |
| .11 | .36 | 359 | 7.25 | 2.02 |
| .06 | .31 | 274 | 4.83 | 1.76 |
| | | Expt. IV | | |
| 0.80 | 1.00 | 1600 | 28.0 | 1.75 |
| .20 | 0.40 | 1555 | 26.9 | 1.73 |
| .11 | .31 | 1635 | 29.0 | 1.77 |
| .06 | .26 | 1083 | 17.0 | 1.57 |
| | | Expt. V | | |
| 0.25 | 0.45 | 1265 | 30.7 | 2.43 |
| .12 | .32 | 1190 | 25.7 | 2.16 |
| .06 | .26 | 546 | 9.55 | 1.75 |
| | | Expt. VI | | |
| 0.25 | 0.45 | 1590 | 35.7 | 2.25 |
| .12 | .32 | 1620 | 34.0 | 2.10 |
| .06 | .26 | 682 | 10.3 | 1.51 |

| Expt. | Dates | Days at $pN_2$ shown in | N at start, mg. |
|---|---|---|---|
| I | 6/16/33–8/6/33 | 30 | 2.06 |
| II | 8/14/33–10/14/33 | 51 | 2.38 |
| III | 2/1/35–3/7/35 | 15 | 1.50 |
| IV | 3/11/35–5/27/35 | 43 | 2.20 |
| V | 9/23/35–12/2/35 | 32 | 2.45 |
| VI | 10/16/35–12/28/35 | 40 | 2.50 |

TABLE II

COMPARISON OF $pN_2$ ON ASSIMILATION OF FREE AND COMBINED NITROGEN BY RED CLOVER

| $pN_2$, atm. | Total pressure, atm. | Dry weight, mg. | Total N, mg. | % N |
|---|---|---|---|---|
| | | Expt. VII, Inoculated | | |
| 0.90 | 1.00 | 732 | 19.4 | 2.65 |
| | | 816 | 22.7 | 2.79 |
| .40 | 0.50 | 958 | 25.1 | 2.62 |
| | | 1002 | 27.1 | 2.71 |
| .20 | .30 | 578 | 15.2 | 2.64 |
| | | 774 | 20.4 | 2.64 |
| .07 | .17 | 394 | 8.9 | 2.26 |
| | | $NH_4NO_3$ | | |
| 0.40 | 0.50 | 1025 | 29.0 | 2.83 |
| .20 | .30 | 889 | 26.6 | 2.99 |
| .07 | .17 | 906 | 32.0 | 3.53 |
| | | Expt. VIII, Inoculated | | |
| 0.90 | 1.00 | 1110 | 24.3 | 2.19 |
| | | 930 | 20.8 | 2.24 |
| .40 | 0.50 | 700 | 14.7 | 2.10 |
| | | 760 | 16.4 | 2.16 |
| .20 | .30 | 860 | 18.3 | 2.13 |
| | | 770 | 17.0 | 2.20 |
| .07 | .17 | 490 | 9.3 | 1.90 |
| | | $NH_4NO_3$ | | |
| 0.90 | 1.00 | 850 | 26.6 | 3.13 |
| .40 | 0.50 | 1220 | 30.0 | 2.46 |
| | | 1130 | 30.6 | 2.71 |
| .20 | .30 | 1020 | 32.3 | 3.16 |
| | | 690 | 23.0 | 3.33 |
| .07 | .17 | 820 | 27.0 | 3.29 |
| | | 1020 | 31.7 | 3.11 |
| | | Expt. IX, Inoculated | | |
| 0.80 | 1.00 | 860 | 18.3 | 2.13 |
| .70 | 1.00 | 880 | 20.3 | 2.31 |
| .20 | 0.50 | 930 | 21.7 | 2.33 |
| .08 | .38 | 610 | 10.8 | 1.77 |
| .04 | .34 | 620 | 7.2 | 1.16 |
| | | $NH_4NO_3$ | | |
| 0.70 | 1.00 | 720 | 29.7 | 4.12 |
| .20 | 0.50 | 830 | 31.5 | 3.80 |
| .08 | .38 | 895 | 33.6 | 3.75 |
| .04 | .34 | 860 | 34.6 | 4.02 |
| | | Expt. X, Inoculated | | |
| 0.80 | 1.00 | 572 | 15.1 | 2.64 |
| .15 | 0.35 | 550 | 13.75 | 2.50 |
| .10 | .30 | 581 | 16.50 | 2.74 |
| .07 | .27 | 497 | 10.35 | 2.08 |
| .04 | .24 | 289 | 5.00 | 1.73 |
| | | $NH_4NO_3$ | | |
| 0.80 | 1.00 | 810 | 24.0 | 2.96 |
| .15 | 0.35 | 920 | 23.6 | 2.57 |
| .10 | .30 | 820 | 24.1 | 2.95 |
| .07 | .27 | 840 | 25.0 | 2.99 |
| .04 | .24 | 830 | 24.2 | 2.91 |

| Expt. | Dates | Days at $pN_2$ shown | N at start, mg. |
|---|---|---|---|
| VII | 7/5/34–9/3/34 | 38 | 1.55 |
| VIII | 9/12/34–11/2/34 | 25 | 3.50 |
| IX | 11/9/34–1/2/35 | 27 | 2.50 |
| X | 12/9/35–2/4/36 | 29 | 2.05 |

ferent total pressures. The results of experiments III and IV show (Table I) that the nitrogen fixation by red clover is independent of the $pN_2$ above 0.11 atm. but if the $pN_2$ is decreased to 0.06 atm. a definite inhibition of fixation occurs. This decrease of fixation with $pN_2$ at pressures below 0.11 atm. was further confirmed in experiments V and VI in which only the lower pressures were included. As shown by the data at the bottom of the table the response is independent of the length of time of experiment (time varied three-fold) or the development of plant at time placed under different $pN_2$ (initial nitrogen content varied two-fold).

In order to demonstrate that the response to $pN_2$ is concerned with the nitrogen fixation process in the plant and not with the general development, it is necessary to demonstrate that growth of uninoculated plants receiving combined nitrogen is completely independent of the $pN_2$. This was done in a series of experiments whose results are summarized in Table II.

Duplicate determinations are given for experiments VII and VIII in order to illustrate the variation found. Because of the limited number of bottles available not all the cultures were made in duplicate in these first experiments, but in later work this was always done, and by improvements in the technique already described, the variation was somewhat reduced. The values given in tables for experiments III to VI and IX to XV are averages of duplicates whose total nitrogen content differed by 10% or less. The data presented in Table II indicate that fixation of nitrogen by clover is essentially independent of the $pN_2$ of the atmosphere until the latter is decreased to a pressure below 0.10 atm. The effect of the $pN_2$ in this lower range is clearly on the fixation process and not on the general development of the plant since the nitrogen content of plants supplied combined nitrogen is independent of the $pN_2$ over the entire range studied.

That the characteristics of the $pN_2$ function do not arise because of growing the plants under reduced pressures was demonstrated in further experiments in which the nitrogen removed was replaced by the inert gases, helium and argon. Since the argon used contained 16% nitrogen, it was not feasible to use partial pressures of nitrogen below 0.16 atm. when this gas was added. Experiments XI, XII and XV were made in 64-oz. bottles, the others in 10-liter bottles. The data in Table III indicate that fixation of elemental nitrogen by inoculated clover plants grown in the presence of helium, argon, or no added inert gas is independent of the $pN_2$ as this is varied from about 0.10 to 0.80 atm. Below a $pN_2$ of 0.10 atm. fixation decreases rapidly with the nitrogen content of the atmosphere. As observed in the other experiments this effect of the $pN_2$ is on the fixation process since plants supplied combined forms of nitrogen assimilate these independent of the $pN_2$ in the atmosphere.

From the data of Table III certain generalizations concerning the symbiotic nitrogen-fixing enzyme system in red clover may be formulated, but before this is done, it is necessary that the essential characteristics of the $pN_2$ function be established definitely. In addition to the experiments reported in the tables, 5 other experiments have been made with essentially the same results. The data from these 20 experiments, 11 of which included combined nitrogen series, were placed on a comparable basis by calculating the *relative* total nitrogen and % N in the plants using the air controls ($pN_2 = 0.80$ atm.) as the base, 100. The plot of the data in Fig. 1 shows that in a given experiment inoculated plants grown under a $pN_2$ greater than 0.10 atm. may contain more or less nitrogen than the air controls, the relative values varying from 80 to 120; the mean value for all the experiments was 90 to 100 for these pressures. On the other hand for $pN_2$ less than 0.10 atm. the relative values range from 35 to 65 with a mean value of 45. Final total nitrogen content of the plants supplied with combined nitrogen exhibited the same type of variation about the 100% value but the mean values were 98 to 104% and apparently independent of the $pN_2$ throughout the range under investigation.

It was noted in the course of these and other experiments that one of the best indicators of inhibition of the fixation process was a decline in the % N in the plant. In some respects this datum is more sensitive to factors which affect the nitrogen fixation system than is total nitrogen. For example, two plant cultures which differ by a comparatively small amount at the start of an experiment are given different treatments. At the end of the test the difference in total nitrogen content appears to be significant, and this would be interpreted as arising from treatment, when actually it is only an expression of the difference in the original cultures. This is a source of error

TABLE III

INFLUENCE OF $pN_2$ ON NITROGEN FIXATION BY CLOVER IN THE PRESENCE OF HE AND A

| $pN_2$, atm. | Gas added | Dry weight, mg. | Total N, mg. | % N |
|---|---|---|---|---|
| | | Expt. XI, Inoculated | | |
| 0.80 | ... | 570 | 13.4 | 2.35 |
| .40 | He | 430 | 8.9 | 2.05 |
| .20 | He | 560 | 13.7 | 2.49 |
| .10 | He | 500 | 11.7 | 2.33 |
| | | $NH_4NO_3$ | | |
| 0.80 | ... | 580 | 19.2 | 3.31 |
| .40 | He | 710 | 25.4 | 3.58 |
| .20 | He | 600 | 18.3 | 3.05 |
| .10 | He | 620 | 17.2 | 2.77 |
| | | Expt. XII, Inoculated | | |
| 0.80 | ... | 424 | 8.90 | 2.10 |
| .20 | He | 379 | 8.13 | 2.15 |
| .11 | He | 300 | 5.64 | 1.89 |
| .06 | He | 268 | 4.32 | 1.61 |
| | | Expt. XIII, Inoculated | | |
| 0.80 | ... | 1455 | 25.15 | 1.73 |
| .25 | ... | 1818 | 26.90 | 1.48 |
| .12 | ... | 1840 | 32.40 | 1.76 |
| .06 | ... | 923 | 13.20 | 1.43 |
| .25 | He | 1780 | 29.80 | 1.67 |
| .12 | He | 1530 | 30.00 | 1.96 |
| .06 | He | 1010 | 16.15 | 1.60 |
| .32 | A | 1465 | 25.9 | 1.77 |
| .16 | A | 1430 | 26.4 | 1.85 |
| | | Expt. XIV, Inoculated | | |
| 0.80 | ... | 310<br>330 | 4.94<br>5.78 | 1.59<br>1.75 |
| .40 | He | 202 | 4.10 | 2.04 |
| .20 | He | 200 | 4.00 | 2.00 |
| .11 | He | 200 | 4.20 | 2.10 |
| .40 | A | 300 | 4.89 | 1.63 |
| .20 | A | 375 | 6.10 | 1.62 |
| | | $NH_4NO_3$ | | |
| 0.80 | ... | 245 | 13.45 | 5.50 |
| .20 | He | 260 | 14.15 | 5.43 |
| .11 | He | 210 | 12.95 | 6.17 |
| .40 | A | 220 | 10.26 | 4.67 |
| .20 | A | 385 | 17.35 | 4.50 |
| | | Expt. XV, Inoculated | | |
| 0.80 | ... | 510 | 15.1 | 2.96 |
| .15 | He | 558 | 14.1 | 2.52 |
| .10 | He | 490 | 12.1 | 2.46 |
| .07 | He | 412 | 9.3 | 2.26 |
| .04 | He | 320 | 7.50 | 2.36 |
| | | $NH_4NO_3$ | | |
| 0.80 | ... | 573 | 19.2 | 3.35 |
| .15 | He | 590 | 19.5 | 3.30 |
| .10 | He | 600 | 20.2 | 3.37 |
| .07 | He | 520 | 18.7 | 3.60 |
| .04 | He | 610 | 18.0 | 2.93 |

| Expt. | Date | Days at $pN_2$ shown | N at start, mg. |
|---|---|---|---|
| XI | 12/22/34–2/11/35 | 24 | 1.05 |
| XII | 2/1/35–3/7/35 | 15 | 1.50 |
| XIII | 6/17/35–9/12/35 | 57 | 3.50 |
| XIV | 4/17/35–6/11/35 | 32 | 1.10 |
| XV | 12/9/35–2/4/36 | 29 | 2.05 |

that must be constantly guarded against in experiments in which final total growths are taken rather than growth rates. However, in these experiments it was noted that even though replicates might vary considerably in total nitrogen (final growth) the percentage nitrogen was much

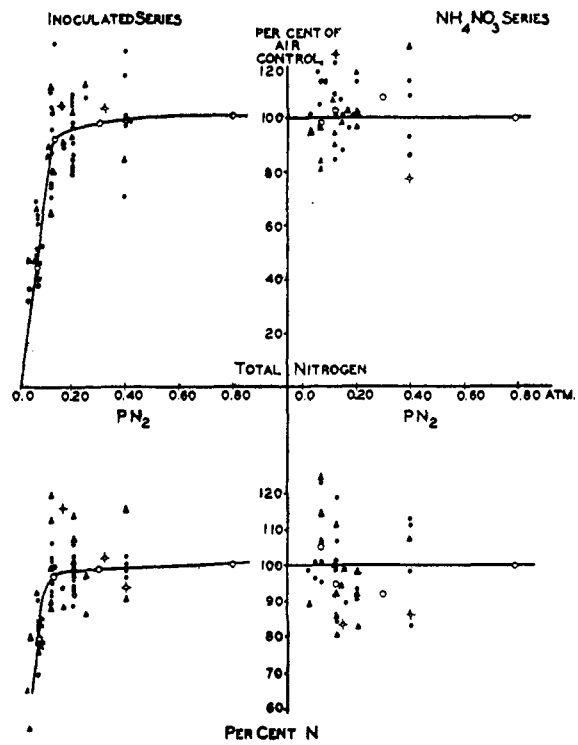


Fig. 1.—Comparison between the $pN_2$ functions of clover plants using free and combined nitrogen. Value for air controls ($pN_2$ = 0.80 atm.) taken as 100 in each case. ●, No added gas; ▲, helium; -☉-, argon; ○, mean of all points in each pressure group.

more stable; frequently the duplicate with the lower total nitrogen had the higher % N, *i. e.*, there is no correlation between total nitrogen and % N if replicates only are considered. On the other hand, under circumstances in which fixation is obviously restricted, *e. g.*, very low $pN_2$, the percentage nitrogen in the plant is definitely decreased. This suggests an additional criterion for distinguishing between those factors which affect the total nitrogen content through specific effect on the fixation system and those which affect the total nitrogen only through effects on the

general growth of the plant. Thus a partial control is available for experiments in which for technical reasons growth rate studies are not particularly suitable.

Plot of the % N in the plants from 20 experiments on a relative basis confirmed the conclusions drawn from the total nitrogen data. As can be seen in Fig. 1 no consistent change in the % N occurs in inoculated plants until the $pN_2$ of the atmosphere is decreased to less than 0.10 atm., then a significant decline is observed. The scatter of the points for pressures greater than 0.10 atm. is usually within ±10% of the 100% line. With plants furnished combined nitrogen, % N in the plants did not appear to change significantly with $pN_2$ over the entire range, although the scatter of the points was greater.

## Discussion

The qualitative observations made from a study of the figures were verified by a statistical analysis of the data from the pooled experiments. The different pressures used were grouped as follows: group 1, 0.04 to 0.07 atm.; 2, 0.10 to 0.12 atm.; 3, 0.20 to 0.40 atm.; 4, 0.80 atm.; and the data from the several experiments subjected to analysis of variance.[5] In the inoculated series 11 experiments were available in which all four pressure groups were represented; in the combined nitrogen series groups 1, 2 and 3 (6 experiments) and groups 2, 3 and 4 (7 experiments) were analyzed separately since all four groups were not represented in all experiments. The analyses show: (1) in the inoculated series the observed differences in total nitrogen and in % N among pressure groups 2, 3 and 4 are not statistically significant, but the differences between group 1 and the remainder are highly significant; (2) in the combined nitrogen series all the observed differences could have arisen from random sampling. The principal statistical constants given in Table IV likewise point to this conclusion.

In consideration of the following statistical analysis of the data, it appears that at pressures of nitrogen greater than 0.10 atm. most of the active points of the enzyme system concerned with the fixation are saturated so that the fixation is independent of the $pN_2$; below this pressure the quantity of nitrogen adsorbed, and hence fixed, is dependent on the $pN_2$.

(5) Fisher, "Statistical Methods for Research Workers," third edition, Oliver and Boyd, London, 1930.

TABLE IV

COMPARISON OF MEANS OF DIFFERENT $pN_2$ GROUPS

| Function | No. of expts. | Pressure, mg. 1[a] | 2 | 3 | 4 | Standard error,[b] mg. |
|---|---|---|---|---|---|---|
| | | Inoculated | | | | |
| Total N | 11 | 10.99 | 20.60 | 22.32 | 22.52 | 1.615 |
| % N | 11 | 1.61 | 1.90 | 1.88 | 1.94 | 0.038 |
| | | $NH_4NO_3$ | | | | |
| Total N | 6 | 31.32 | 33.02 | 32.93 | ... | 2.770 |
| | 7 | ... | 20.80 | 22.38 | 20.73 | 1.315 |
| % N | 6 | 3.28 | 3.03 | 2.85 | ... | 0.158 |
| | 7 | ... | 3.42 | 3.33 | 3.69 | .183 |

[a] Pressures: 1, 0.05–0.07 atm.; 2, 0.10–0.12 atm.; 3, 0.20–0.40 atm.; 4, 0.80 atm.

[b] Of difference between two means, *i. e.*, difference between two means must be twice this before it can be regarded as significant.

Because of variation in the plants it is not possible to characterize definitely the exact nature of the curve in the region in which complete saturation is replaced by partial saturation since relatively large differences are necessary in order that they fall outside the limits of experimental error. Likewise, control of the $pN_2$ under the experimental conditions described is probably restricted to about ±0.01 to 0.02 atm. In spite of the limitations the data in Table IV show that it is improbable (38:1 odds): (1) that the true mean of the 0.10–0.12 atm. series is less than 18.32 mg. (observed mean minus twice standard deviation) and (2) that the true mean of the air control groups is greater than 24.80 mg. This means that under the most unfavorable assumptions, a 75% saturation at $pN_2$ of 0.10 to 0.12 atm. is indicated. Considerably higher saturation, (up to 100%) is more probable; this is borne out by consideration of the % N data. The curves for the inoculated series in Fig. 1 were drawn through the mean values for each pressure group with the result that the transition regions are not well defined; it is not improbable that the true curve of the $pN_2$ function approximates a typical Langmuir adsorption isotherm.

Burk[2,6] on the basis of both total growth (respiration) and rate of respiration has shown that fixation of nitrogen by the *azotase* complex in the free living nitrogen-fixing bacteria *Azotobacter* is a function of the $pN_2$ until the latter becomes relatively large—up to 5 to 10 atmospheres—and that the rate is almost directly proportional to the $pN_2$ up to 0.5 atm. The dissociation constant ($K_{N_2}$, equivalent to substrate concentration at which rate is 50% of maximum) of the enzyme–nitrogen

(6) Burk, *J. Phys. Chem.*, **34**, 1195 (1930).

complex was found to be 0.215 ± 0.002 atm., a value which Burk states is somewhat high in comparison with other gases for different enzyme systems. In our own work the data observed were total growth and not growth rates, hence $K_{N_2}$ cannot be precisely determined from these values. However, since under the experimental conditions used, the rate will roughly follow the final observed total growth, an approximation of $K_{N_2}$ is possible. It would appear that $K_{N_2}$ for the symbiotic system is below 0.10 atm. possibly in the neighborhood of 0.05 atm. Whether this difference between the dissociation constants represents a real difference in these two biological nitrogen-fixing systems is still open to question, but further experiments concerned with the character of the enzyme complex should be able to supply an answer.


## Summary

The relation of the partial pressure of nitrogen to fixation of the free element by the symbiotic system in red clover has been studied over the range of $pN_2$ from 0.04 to 1.56 atm. in the presence of no added gas, helium and argon. Statistical analyses of data from 11 experiments indicate that the fixation of nitrogen is essentially independent of $pN_2$ when the latter exceeds 0.10 to 0.20 atm. but decreases rapidly with $pN_2$ as the latter is diminished below 0.10 atm. This $pN_2$ function is characteristic of the nitrogen fixing process since it is not observed in plants supplied with combined nitrogen. The implications of these observations for the mechanism of the symbiotic fixation process are discussed.


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# The Mechanism of the Dehydration of Calcium Sulfate Hemihydrate

BY HARRY B. WEISER, W. O. MILLIGAN AND W. C. EKHOLM

During the past few years several papers have appeared which reopen the question of the chemical individuality of the so-called calcium sulfate hemihydrate or plaster of Paris and the mechanism of its dehydration. Linck and Jung[1] deduced from dehydration data that plaster of Paris loses its water after the manner of zeolites. Balarew[2] at different times has been on all sides of the question. At first[2a] he claimed that the dehydration isobar would indicate that the water is present in the form of a true hydrate; later[2b] he stated that the dehydration curves show the water to be held in a new way "half-hydratic and half-zeolitic"; finally,[2c] he concluded that the water is lost in the manner of zeolites. Gibson and Holt[3] concluded from pressure–temperature curves that the water is lost continuously as in a zeolite. Parsons[4] claimed that all the water in gypsum may be lost by heating without the intermediate formation of a hemihydrate.

Jung,[5] Ramsdell and Partridge[6] and Caspari[7] reported that the x-ray diffraction pattern of calcium sulfate hemihydrate and its dehydration product[8] are identical, in agreement with the view that the hemihydrate is a zeolite. Onorato[9] and Gallitelli[10] found the hemihydrate and dehydrated hemihydrate to have the same type of structure but recognized minor differences in the powder x-radiograms. Feitknecht[11] likewise observed differences in the two patterns provided care was taken to prevent rehydration of the dehydrated hemihydrate. Feitknecht believed that Jung's dehydrated sample must have rehydrated before x-ray examination was made.

From the above survey it appears to be an open question whether "calcium sulfate hemihydrate" is a true chemical hydrate which gives a definite x-radiogram and decomposes to "dehydrated hemihydrate" which gives a different x-radiogram

(1) Linck and Jung, *Z. anorg. allgem. Chem.*, **137**, 407 (1924).

(2) (a) Balarew, *ibid.*, **156**, 258 (1926); (b) *Kolloid.-Z.*, **48**, 63 (1929); (c) Balarew and Koluschewa, *ibid.*, **70**, 288 (1935).

(3) Gibson and Holt, *J. Chem. Soc.*, 638 (1933).

(4) Parsons, Univ. Toronto Studies, Geol. Ser., No. 24, 1927, p. 24.

(5) Jung, *Z. anorg. allgem. Chem.*, **142**, 73 (1925).

(6) Ramsdell and Partridge, *Am. Mineral.*, **14**, 59 (1929).

(7) Caspari, *Nature*, **133**, 648 (1934).

(8) It should be pointed out that van't Hoff, Henrichsen and Weigert [*Sitz. akad. Wiss.*, 570 (1901)] prepared a "soluble" form of calcium sulfate by treating gypsum with nitric acid containing 2.38 moles of water per mole of nitric acid, above 50°. This material was named "soluble anhydrite" to distinguish it from the ordinary "insoluble" anhydrite. Although the term "soluble anhydrite" is usually applied to the product formed by the dehydration of calcium sulfate hemihydrate below 200°, Balarew[2b] believes this to be distinct from the "soluble anhydrite" of van't Hoff. Both Balarew and Ramsdell and Partridge[6] prefer to apply the term "dehydrated hemihydrate" to the dehydration product, and this terminology is used in this paper.

(9) Onorato, *Periodico Min.*, **3**, 138 (1932).

(10) Gallitelli, *ibid.*, **4**, 1, 132 (1933).

(11) Feitknecht, *Helv. Chim. Acta*, **14**, 85 (1931).