pletely detached. In contrast with an earlier report that similar treatments gave rise to large increases in photosynthetic rates in cucumbers, little or no stimulation of respiration or photosynthesis was produced by any of these treatments. Instead, there appears to be a gradual decline in both photosynthesis and respiration.

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INFLUENCE OF COBALT ON NITROGEN FIXATION BY MEDICAGO [1]

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Reisenauer (6) has discussed the reasons for exploring the possibility that cobalt may be implicated in symbiotic nitrogen fixation. Data were also presented which demonstrated the essential role of cobalt in the functioning of the Rhizobium-alfalfa (Medicago sativa L.) system in nitrogen fixation.

The present paper presents additional data obtained during the early winter season (Oct.–Dec. 1959) under somewhat more favorable greenhouse conditions and with some other modifications. As a consequence yield differences reflecting efficiency of nitrogen fixation with and without cobalt supplements are considerably greater than those reported earlier for summer (May–July 1959) experiments, as well as those obtained by Ahmend and Evans with Glycine max Merr. (1). Also, numerous requests for more detailed information on preparation of growth media sufficiently low in cobalt to permit observations of direct positive influence of the element on nitrogen fixation make it seem desirable to elaborate in detail on our experimental procedures in this paper.

MATERIALS & METHODS

WATER SUPPLY: All water used in cleaning glassware, including solution storage bottles and culture vessels, in preparing nutrient media, and in making up water during the growth period was of high purity as determined by the dithizone test for heavy metals. Distilled water prepared by the usual procedures in an all block-tin system was again distilled from a still provided with a borosilicate glass head and condenser. Water was stored in borosilicate glass bottles.

GLASSWARE: All glassware, of borosilicate glass, was rigorously cleaned by washing with 0.1 M trisodium ethylenediamine tetraacetic acid (EDTA) solution, rinsing with redistilled water, followed by washing with 1 M HNO₃, and rinsing again with redistilled water. Culture vessels were 4-liter beakers with covers of plaster of paris coated with paraffin.

PURIFICATION OF SALTS USED IN NUTRIENT SOLUTIONS. Earlier experiments designed to test the cobalt requirements of tomatoes indicated that cobalt could be successfully removed from salt solutions by alkaline sulfide precipitation with copper as carrier (3). However, further study of the procedure

(Frinck, C. R. MS thesis U.C., 1957) cast doubt on the general reproducibility of these purification procedures. Therefore other procedures were investigated, using Co^{60} as an indicator of efficiency of purification.

The method adopted for these experiments was based on coprecipitation with iron oxinate as described by Hewitt (5) with some modification.

Volatile acids, ammonia, and organic solvents were distilled before use. Solutions of 2 M $Ca(NO_3)_2$, 1 M K H_2PO_4, and 0.5 M K_2SO_4 were prepared. Twenty milliliters of a 5 % oxine solution in 2 M acetic acid and 5 ml of 0.2 M $FeCl_3$ were added to each liter of salt solution. Ammonium hydroxide (approx 6 M) was added to each solution to a pH of 5.1 to 5.2. After standing overnight, the solution was filtered through thoroughly washed filter paper and the precipitation step repeated. Excess oxine was extracted from the second filtrate with chloroform. Finally the chloroform was removed from the solutions by several extractions with diethyl ether. Residual ether was removed by heating the solutions on the steam bath.

Calcium sulfate was prepared by mixing equimolar quantities of the purified calcium nitrate and potassium sulfate. The precipitate was washed with redistilled water until free of nitrate as shown by a diphenylamine test of the washings.

Calcium carbonate was prepared by passing carbon dioxide and ammonia gas into a solution of purified calcium nitrate, keeping the pH in the 8 to 9 range. The precipitate was freed of nitrate by washing with redistilled water.

Magnesium sulfate was purified by extracting a 1 M solution at pH 8.5 with 0.01 % dithizone in carbon tetrachloride. Three successive 100 ml portions of the dithizone solution were used to extract the heavy metals from 1 liter of magnesium sulfate. If the magnesium sulfate contains large amounts of dithizone-extractable metals more extractions may be necessary. Excess dithizone was removed from the aqueous magnesium sulfate solution by repeated extractions with chloroform. Soluble chloroform was removed by extracting with diethyl ether and the ether was removed by heating on the steam bath.

Iron was freed of cobalt by extracting a $FeCl_3$-6 M HCl solution with diethyl ether which had been saturated with 6 M HCl. As shown by Grahame and Seaborg (4) with radiocobalt and an iron to cobalt ratio of 10^8:1, separation of iron and cobalt is quantitative. The ether and excess HCl were removed by evaporation of the solution to a dense syrup on the steam bath. The $FeCl_3$ was made to standard volume and an aliquot analyzed for iron prior to preparation of the chelated micronutrient solution.

Other micronutrients: Manganese chloride and the sulfates of zinc and copper were twice crystallized. Boron was supplied from twice crystallized boric acid. Chloride requirement for plants was met by the chloride associated with ferric chloride and manganese chloride.

TABLE I

MICRONUTRIENT STOCK SOLUTION

| | 1 ml STOCK/L OF CULTURE PROVIDES FOLLOWING CONCS OF ELEMENTS | | |
SALT	ELEMENT	μg ATOMS/L	PPM APPROX
H_3BO_3	B	25	0.27
$MnCl_24H_2O$	Mn	5	0.27
	Cl	10	0.36
$ZnSO_47H_2O$	Zn	2	0.13
$CuSO_45H_2O$	Cu	0.5	0.03
Na_2MoO_4	Mo	0.5	0.05
$FeCl_3$	Fe	90	5.0
	Cl	270	9.5
EDTA	...	97.5	...

EDTA was purified by dissolving the acid in dilute ammonium hydroxide and reprecipitating it by the addition of HCl. This procedure was repeated twice. The acid was dried under vacuum at 70° C for 24 hours to provide the anhydrous form.

Chelated micronutrient stock: The micronutrient solution was prepared in such concentration that 1 ml per liter of culture solution provided the concentration of micronutrients indicated in table I. Sufficient EDTA was used to provide an equimolar ratio with the micronutrient cations. Redistilled ammonium hydroxide was added to neutralize the acidity of the EDTA and $FeCl_3$ to a final pH of 5.5. The solution was stored in a dark bottle at 4° C.

The concentrations of macronutrients are given in table II. One-fourth of the indicated concentration was present in the solution at the time of planting the seedlings. After 10 days the remainder was added. In addition, all treatments received an initial application of 1.25×10^{-4} moles of $Ca(NO_3)_2$ per liter to provide a limited amount of nitrogen until symbiotic nitrogen fixation became effective. When differential treatments were applied (10 days after planting)

TABLE II

MACRONUTRIENT SOLUTION COMPOSITION

| SALT SOURCE | mg ATOMS OF ELEMENT/L NUTRIENT | | | | |
	Ca	Mg	K	S	P
KH_2PO_4	...	...	2	...	2
K_2SO_4	...	...	4	2	...
$MgSO_4$	...	1	...	1	...
$CaCO_3$	2	...	...	...	...
$CaSO_4$	2	...	...	2	...
Total mg atoms/l	4	1	6	5	2
Total ppm	160	24	235	160	62

two sets of plants were placed on a high nitrogen regime, receiving 5×10^{-3} moles of $Ca(NO_3)_2$ per liter of culture (see table III). $CaCO_3$ and $CaSO_4$ were not added to these beakers because calcium was supplied by the $Ca(NO_3)_2$ addition.

Solutions were not changed during the 8-week experimental period. Occasional checks of pH of the nutrient were made with pH values ranging from 6.0 to 6.5. No adjustment of pH was made.

Alfalfa seedlings, germinated on cheesecloth in 1/20 strength nutrient solution in glass trays, were transferred to the culture vessels when 10 days old. The culture vessels were covered with double thickness jackets (blue denim inside, white outside) to exclude light from the solution. Continuous aeration was employed.

INOCULATION: Week-old cultures of *Rhizobium meliloti* from mannitol-yeast extract agar plates were used in preparing the suspension for inoculation. Removal of possible cobalt contamination in the mannitol-yeast extract agar media was not attempted. Reliance was placed on the high order of dilution of the microorganisms with high purity water in minimizing cobalt contamination. One milliliter of a suspension containing approximately 10^4 organisms was added to each 4-liter culture 10 days after the plants were placed in the solutions. Two weeks later, because nodulation was very sparse, an additional inoculation of approximately 10^6 organisms per 4 liters was made. Within a week nodulation was profuse.

TRACER STUDIES: For an experiment with isotopic nitrogen, excised nodulated roots from treatment A (minus cobalt) and from treatment B (plus cobalt) were cut into approximately 3-cm lengths and 5-g samples of each were added to Thunberg tubes. Samples were incubated in the presence of isotopic nitrogen gas. After a suitable incubation period the root tissue was subjected to Kjeldahl digestion and N^{15} was determined on the ammonium ion obtained therefrom by means of a ratio-type mass spectrometer.

TABLE III

DIFFERENTIAL TREATMENTS

| TREATMENT DESIGNA-TION | VARIABLES | | RHIZOBIUM INOCULA-TION |
	NITRATE CONC	Co (0.1 μmoles/1)	
A	1.25×10^{-4} M	—	+
B	1.25×10^{-4} M	+	+
C	1.25×10^{-4} M	+	—
D	1.25×10^{-4} M	—	—
E	51.25×10^{-4} M	—	—
F	51.25×10^{-4} M	+	—

EXPERIMENTAL DESIGN

Three beakers of 4-liter volume, each containing 5 alfalfa (*Medicago sativa*) plants, were used for each of six treatments. All treatments had an initial supply of nitrate nitrogen of 1.25×10^{-4} moles per liter. Ten days after transfer of seedlings to the 4-liter beakers the differential treatments were imposed. Treatments are shown in table III.

RESULTS

Treatments of principal interest are A and B, Rhizobium inoculated without and with supplemental cobalt, respectively. Treatments C and D were included to observe the extent of nitrogen deficiency obtaining during the growth period with the restricted fixed nitrogen supply (1.25×10^{-4} M) and no intentional inoculation with Rhizobium. The yields (table IV) obtained in these treatments served as reference points for judging the effect of cobalt on nitrogen fixation in inoculated systems. One beaker of treatment C was accidentally inoculated the second time. The error was noted immediately and this beaker was then designated as a treatment B. Thus treatment B

TABLE IV

EFFECT OF COBALT & RHIZOBIA ON GROWTH OF ALFALFA

| TREATMENT* | FRESH WT YIELD—GRAMS PER CULTURE | | | |
| | TOPS | | ROOTS | |
	MEAN WT	S_x**	MEAN WT	S_x
A Nodulated — no cobalt	12.7	0.569	38.0	1.05
B Nodulated + cobalt	42.5	2.17	36.2	1.69
C Non-inoculated + cobalt	10.1	0.90	33.0	1.50
D Non-inoculated — no cobalt	11.1	0.292	32.7	1.11
E $Ca(NO_3)_2$ — no cobalt	101.2	8.94	96.3	14.1
F $Ca(NO_3)_2$ + cobalt	96.3	5.07	92.0	13.0

* All beakers received an initial supply of nitrate, at the rate of 1.25×10^{-4} moles per liter. Treatments E and F received additional Ca $(NO_3)_2$ at rate of 5×10^{-3} moles per liter 10 days after the start of the experiment. Plus cobalt treatments received 0.1 μmoles $CoSO_4$ per liter of cultures.

** Standard error of mean.

then consisted of four beakers and treatment C of only two. No nodules were found on plants which were not inoculated.

Treatments E and F are of interest only in reference to the effect of cobalt supplements on yield of alfalfa when supplied with fixed nitrogen. As will be noted later, no specific differences between these two treatments were detected beyond the limits of experimental variations.

Comparisons of yields from beakers supplied with nitrate nitrogen (treatments E & F) with those of beakers where symbiotic nitrogen fixation was operative (treatment B) cannot be made because of difficulties in timing of application of nitrate nitrogen to coincide with initiation of symbiotic nitrogen fixation. It had been planned to add nitrate at the time of appearance of first nodules. However, failure of the first inoculation to provide a reasonable number of nodules required a second inoculation with consequent time delay. Thus, plants of treatments E and F made considerably greater growth than those of treatment B because of a nearly 3-week earlier supply of nitrogen in excess of that provided initially.

A summary of chronology of the experiment may be useful in this regard. Seedlings germinated October 5, 1959; transplanted to dilute nutrient (including 1.25×10^{-4} moles $Ca(NO_3)_2$/liter) on October 15, 1959; full complement of nutrient salts added (except $Ca(NO_3)_2$) to all treatments October 26, 1959; 5×10^{-3} moles $Ca(NO_3)_2$ per liter added to treatments E and F October 26, 1959; $CoSO_4$ (1×10^{-7} moles/liter, approx 0.006 ppm Co) added to plus cobalt treatments October 26, 1959; first inoculation with 10^4 cells of *Rhizobium meliloti* October 26, 1959; second inoculation with 10^6 cells of *Rhizobium meliloti* November 9, 1959; nodulation profuse and green color returning to nitrogen deficient plants of treatment B by November 16, 1959; harvested all plants December 11, 1959.

Note that plants of treatments E and F never became nitrogen deficient because of supply of $Ca(NO_3)_2$. Plants of all other treatments showed clear symptoms of nitrogen deficiency by November 13, 1959. The plants of treatment B regained their green leaf color by November 16, 1959. All plants of other treatments remained nitrogen deficient as judged by pale green leaf color and purpling of stems. Dry weights, nitrogen data and top-to-root ratios are presented in table V. No dry weight figures are available for the roots of treatments A and B, inasmuch as the tissues were utilized for studies with isotopic nitrogen.

It was desirable to determine whether or not there was any directly demonstrable difference in the nitrogen fixing ability of nodulated roots grown in the presence and in the absence of cobalt, as well as to observe the effect on nitrogen fixation of adding cobalt to previously deprived roots and nodules. Nodulated root sections were placed under isotopic nitrogen in Thunberg tubes as indicated in table VI. Samples were incubated for various times as shown in the table and isotopic nitrogen was recovered in the ammonia fraction from Kjeldahl digestion of these tissues. Tissues grown in the presence of added cobalt ion were significantly more active in their capacity for nitrogen fixation. Moreover, tissues grown in the absence of added cobalt ion were able to fix nitrogen provided cobalt was added immediately before the period of administration of isotopic nitrogen. Adding glucose appeared to have little if any effect on accelerating the fixation of nitrogen. It is evident by reference to the various time treatments that most of the nitrogen fixed by the excised root segments was fixed during the first 4 hours of incubation. This finding is in keeping with the observation of Aprison and Burris (2) that the capacity for nitrogen fixation by excised nodules of Glycine fell off rapidly with time after the nodules were removed from the plant.

DISCUSSION

From the above data (tables IV & V) it can be seen that the combination of cobalt and an inoculum of Rhizobium make it possible for plants of *Medicago sativa* to grow luxuriantly under conditions which

TABLE V
DRY WEIGHTS & NITROGEN VALUES

a TREAT-MENT*	Tops				Roots				h TOP-ROOT RATIO d/g
	b MEAN DRY WT g	c MEAN N CONTENT mM/g	d NITROGEN CONTENT b x c	S_x	e MEAN DRY WT g	f MEAN N CONTENT mM/g	g NITROGEN CONTENT e x f	S_x	
A	3.0	0.78	2.34	0.27	**	**	**	...	...
B	7.3	2.79	20.4	0.34	**	**	**	...	...
C	2.3	0.93	2.14	0.28	4.7	0.73	3.44	0.40	0.62
D	2.5	0.92	2.30	0.05	4.9	0.70	3.44	0.20	0.67
E	18.9	1.83	34.6	2.4	14.1	1.00	14.1	2.8	2.45
F	18.0	1.95	35.1	2.1	12.5	1.50	18.7	2.1	1.78

* See table IV.
** Not available. Fresh tissues were used for N^{15} studies.

TABLE VI

FIXATION OF NITROGEN BY NODULATED ROOT SEGMENTS

TUBE NO.	ROOT MATERIAL	INCUBATION TREATMENT	HR INCUBATION WITH N^{15}	TOTAL N (μmoles)	N^{15} CONTENT (ATOM % EXCESS)	N FIXED (μmoles)
1	Treatment A (minus cobalt)	No added cobalt	24	145	0.003	0.0043
2	,,	,, ,, ,,	24	143	0.003	0.0043
3	,,	2×10^{-8} Molar $CoSO_4$	24	146	0.018	0.026
4	,,	4×10^{-8} ,, ,,	24	119	0.016	0.018
5	,,	6×10^{-8} ,, ,,	24	152	0.016	0.024
6	,,	10^{-7} ,, ,,	2	120	0.010	0.012
7	,,	,, ,, ,,	4	170	0.010	0.017
8	,,	,, ,, ,,	8	182	0.010	0.018
9	,,	,, ,, ,,	24	185	0.011	0.020
10	,,	,, ,, ,,	48	186	0.011	0.020
11	Treatment B (plus cobalt)	,, ,, ,,	2	576	0.014	0.081
12	,,	,, ,, ,,	4	496	0.026	0.129
13	,,	,, ,, ,,	8	572	0.036	0.206
14	,,	,, ,, ,,	24	440	0.035	0.154
15	,,	,, ,, ,,	48	554	0.036	0.198
16	,,	,, ,, ,, (no added glucose)	24	477	0.048	0.226
17	,,	,, ,, ,, (no added glucose)	11	572	0.035	0.200

Except as indicated above each tube received 5 grams nodulated root segments, 1 ml 1 % glucose, 10 ml cobalt-free culture solution (treatment A, table IV) and 1 ml 10^{-6} M $CoSO_4$. Tubes were evacuated and refilled with 2 cm 100 atom % N^{15} (40 micromoles) and 50 cm O_2 (1 millimole).

otherwise would produce severe nitrogen deficiency. Absence of either cobalt or Rhizobium inoculum resulated in severe nitrogen deficiency. It should perhaps be pointed out that the initial provision of 1.25 × 10^{-4} moles of calcium nitrate per liter of culture solution was necessary in order to obtain experimental material with which to work. In spite of this the quantitative figures in terms of fresh weight yield of plant material as a result of the addition of cobalt are striking, with greater than a threefold increase in yield during the 4-week nitrogen stress period. From a qualitative standpoint the difference between plants was even more noticeable. This is due in a large part to the fact that there was essentially no observable difference in the amount of root material produced under the various test conditions (excepting for the greater yield of roots obtained from those plants furnished with additional calcium nitrate (treatments E & F) as explained above. This relationship is further borne out by an inspection of the data given in table V showing nitrogen values and top-to-root ratios resulting from the various treatments.

Not expressed by these data but observable visually as the experiment progressed, was an apparent tendency of the plants to overcome to a limited extent the nitrogen deficiency resulting from a lack of cobalt. There appeared to be a slight greening of the cobalt

deficient plants just prior to harvest. There are a number of possible explanations for this observation, including the possibility that a small but significant quantity of cobalt was being added with replacement water. It is also possible that nodulated plants have the ability to fix nitrogen independent of the presence of added cobalt, after a lag period.

Although the above data clearly indicate a dependence of the fixation reaction upon cobalt ion, it is not clear where that ion has its effect in the physiological and biochemical chain of events leading finally to the production of fixed nitrogen in the plant. There is some evidence, circumstantial in nature, which suggests that the presence or absence of cobalt did not influence the growth of Rhizobium nor the process of nodulation, but was more directly concerned with the nitrogen fixation reaction itself, or with the synthesis of enzymes or co-enzymes necessary for that reaction. This is based in part on the observation that there was extensive nodulation, and presumably therefore growth of Rhizobium, in the minus cobalt treatments. No attempt was made to study the nodules in section nor to determine the number of organisms present in them. Perhaps the strongest argument favoring the postulation of a direct role of cobalt in the fixation reaction is the immediate effect which adding cobalt had on the fixation of isotopic

nitrogen by excised nodulated root sections.

The studies with excised root sections also indicate that the capacity for fixation is lost soon after the roots are removed from the plant. This suggests that some metabolite produced in the tops is essential for fixation.

Summary & Conclusions

I. Alfalfa plants (*Medicago sativa*) deprived of sufficient inorganic nitrogen were grown in the presence and absence of added cobalt ion and an inoculum of an appropriate strain of Rhizobium.

II. With added cobalt ion and a Rhizobium inoculum, significantly greater yields were obtained than those observed in the absence of such treatment.

III. Cobalt ion alone or a Rhizobium inoculation alone produced no such enhanced growth over control treatments.

IV. The effect of such treatment on root development was negligible during the period of the experiment, but large differences in top growth were observed.

V. Using isotopic nitrogen, roots and nodules grown in the presence of added cobalt possess a greater capacity for nitrogen fixation than those grown without added cobalt.

VI. When cobalt-deficient nodulated root sections were placed in culture solutions containing as little as 2×10^{-8} molar cobalt ion, nitrogen was fixed within a short period.

VII. With isolated root sections carrying nodules, most of the nitrogen was fixed within the first 2 hours of incubation.

VIII. It is concluded that cobalt ion plays a major role in the Medicago-Rhizobium symbiosis.

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