THE EFFECT OF COBALT ON THE GROWTH OF RHIZOBIUM JAPONICUM[1]

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Cobalt in small amounts has been shown to cure certain deficiency symptoms in ruminant animals (Underwood 1956) and also is essential for the normal growth of blue-green algae (Holm-Hansen, et al. 1954). It is apparent from the literature that cobalt plays a major role in metabolism as a constituent of vitamin B_{12}; however, the essentiality of cobalt for most of those microorganisms known to synthesize the vitamin has not been demonstrated (Ford and Hutner 1955). Recent investigations (Ahmed and Evans 1959) have shown that the addition of cobalt to cultures of soybean plants grown under symbiotic conditions resulted in marked increases in the dry weight of shoots and prevented the development of nitrogen deficiency symptoms that were apparent on plants that were not provided with the element. The experiments of Reisenauer (1960) have provided evidence that the growth and nitrogen content of symbiotically grown alfalfa is strikingly stimulated by cobalt. Further investigations (Ahmed and Evans 1960) have shown conclusively that cobalt is an essential element for the growth of soybean plants under symbiotic conditions, but no response could be demonstrated in experiments where adequate fixed nitrogen was supplied. It was concluded that cobalt must play a role in the metabolism of Rhizobium and or in the symbiotic relationship of the Rhizobium and the soybean plant. These conclusions prompted initiation of a study of the effect of cobalt on Rhizobium japonicum grown in pure culture.

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The methods used for the purification of water and mineral salts were those described by Ahmed and Evans (1960). For the purification of arabinose and inositol, the 1-nitroso-2-naphthol extraction procedure of Bolle-Jones and Mallikarjuneswara (1957), was employed. The solutions of arabonose (0.4 g per ml) and inositol (30 mg per ml) were mixed with approximately 16 ml of 1-nitroso-2-naphthol (0.12 percent in 0.05 percent aqueous NaOH) for each 1 of solution. The solutions were warmed to 40°C and after cooling each 1 of solution was extracted with 80 ml of redistilled chloroform. The extractions were repeated twice. Solutions were treated with 0.25 g of acid washed charcoal (Ahmed and Evans 1960) and then were filtered through acid washed filter papers. Commercial vitamins were used without further purification. Cobalt was added as a solution of $CoCl_2$ prepared by dissolving spectrographically standardized metal in a small quantity of redistilled HCl and diluting to the desired concentration with demineralized water. All of the glassware used was acid washed with 3 N HCl and throughly rinsed with deionized water.

The basic nutrient medium contained the following per 1: K_2HPO_4, 1.00 g; KH_2PO_4, 1.10 g; $MgSO_4 \cdot 7H_2O$, 0.31 g; $Ca(NO_3)_2 \cdot 4H_2O$, 1.16 g; $FeCl_3$, 0.48 mg; $ZnSO_4 \cdot 7H_2O$, 0.18 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.23 mg; H_3BO_3, 0.28 mg; $MnSO_4 \cdot 4H_2O$, 0.23 mg; $CuSO_4 \cdot 5H_2O$, 0.016 mg; L arabinose, 4.0 g; inositol, 4.8 mg; thiamine hydrochloride, 0.1 mg; riboflavin, 0.1 mg; p-aminobenzoic acid, 0.1 mg; nicotinic acid, 0.1 mg; pyridoxine hydrochloride, 0.1 mg; and calcium pantothenate, 0.1 mg. The purified $FeCl_3$ solution added to each 1 of the medium contained 0.04 equivalents of HCl. The pH of the culture medium was 6.6.

Turbidity measurements were made with a Beckman Model B spectrophotometer at a wavelength of 600 mμ. The sterile culture medium without inoculation was used as a blank. Bacterial cells from each culture flask were collected and washed by centrifugation and then their nitrogen contents were determined by a micro Kjeldahl procedure (Johnson 1941).

An effective nitrogen-fixing strain of Rhizobium japonicum used in the experiments was obtained from Dr. L. W. Erdman of the U. S. Department of Agriculture and was maintained on agar slants containing yeast extract and mannitol. The organism was transferred to 50 ml flasks each containing 20 ml of sterile, purified medium. After incubating for 4 days at 30°C on a reciprocating shaker, 0.05 ml of the culture was transferred to each of a series of 250 ml flasks containing 50 ml of the sterile, purified medium with the cobalt additions indicated in Experiment I of Table I. In Experiment II (Table I) 0.1 ml of inoculum was used for each flask; otherwise, the two experiments were identical. The turbidities and nitrogen contents of the cultures were measured after growth periods indicated in Table I.

TABLE I

Effects of Cobalt on the Growth of Rhizobium japonicum

Treatment	Turbidity		Nitrogen Content**
	5 days	7 days	
	O.D.	O.D.	mg/flask
Experiment I			
0 cobalt*	0.02	0.35	0.53
0.5 ppb. cobalt	0.19	1.27	4.75
5.0 ppb. cobalt	0.21	1.40	5.00
Experiment II			
0 cobalt*	--	0.39	0.62
0.5 ppb. cobalt	--	1.33	5.50
5.0 ppb. cobalt	--	1.38	5.12

* Values for the 0-cobalt treatment in both experiments are means of determinations on duplicate cultures. Other measurements were made on single cultures.

** Nitrogen contents of cells were determined after a growth period of 7 days.

The data in both experiments indicate that the addition of either 0.5 or 5 ppb (parts in 10^9 parts) cobalt to flasks resulted in marked increases in the growth of the bacteria as measured by turbidity. The total nitrogen

content of the cells from cobalt-treated cultures in both experiments was about 10-fold that of cells from cultures lacking cobalt.

It seems apparent from these results and from those presented previously that cobalt is an important growth factor for Rhizobium japonium. The evidence obtained from experiments utilizing soybean plants inoculated with Rhizobium japonium and cultured under symbiotic conditions have shown that cobalt is highly specific for the symbiotic growth of Rhizobium and soybean plants. From the results presented here, it seems that cobalt may play a major role in the bacteria regardless of whether it is grown with or without the leguminous plant. Further investigations concerning the specificity of cobalt for the growth of Rhizobium and the relationship of the element to vitamin B_{12} synthesis are in progress.

References

Bolle-Jones, E. W. and Mallikarjuneswara, V. R., J. Rubber Res. Inst. Malaya, 15:128, 1957.

Ford, J. E. and Hutner, S. H., in Vitamins and Hormones, 13:101, edited by Harris, Robert, Marrian, B. F., and Thimann, K. V., Academic Press, New York, 1955.

Holm-Hansen, O., Gerloff, G. C., and Skoog, F., Physiol. Plant., 7:665, 1954.

Johnson, M. J., J. Biol. Chem., 137:375, 1941.

Reisenauer, H. M., Nature, 186:375, 1960.

Shaukat-Ahmed and Evans, Harold J., Biochem. and Biophys. Research Communications, 1:271, 1959.

Shaukat-Ahmed and Evans, Harold J., Soil Sci., 90:205, 1960.

Shaukat-Ahmed and Evans, Harold J., accepted by Proc. Nat. Acad. Sci., 1960.

Underwood, E. J., Trace Elements in Human and Animal Nutrition, Academic Press, Inc., New York, 1956.