The effect of cobalt deficiency on the utilization of nitrate nitrogen in Rhizobium

AHMED AND EVANS[1] established that cobalt is essential for soyabean plants grown under symbiotic conditions and REISENAUER[2] obtained similar results with alfalfa. The metal requirement was, however, abolished when the plants were supplied with combined nitrogen. LOWE, EVANS AND AHMED[3] have now shown that cobalt is essential for the growth of *Rhizobium japonicum* in medium containing nitrate as the sole source of nitrogen.

In this communication we report that a deficiency of cobalt in *Rhizobium japonicum* (strain 505) markedly reduced nitrate reductase activity in whole cells and their extracts when either sodium succinate, DPNH or reduced benzyl viologen was used as a hydrogen donor for the enzyme. By incorporating ^{58}Co in the culture medium it was found that the micronutrient did not concentrate in purified fractions of the enzyme so that the effect of cobalt deficiency may well be indirect.

Rhizobium japonicum (strain 505) kindly supplied by Mrs. HARRIS, School of Agriculture, Nottingham University, was grown in the following culture medium containing per litre: KNO_3, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; K_2HPO_4, 0.17 g; mannitol, 5 g. Cobalt was removed from a solution of these constituents using the 1-nitroso-2-naphthol method[3]. The following spectrographically pure micronutrients were added to the purified culture media: Cu, 25 μg/l; Mn, 600 μg/l; Zn, 90 μg/l; Fe, 500 μg/l. Biotin and thiamine hydrochloride at 10 μg/l and 100 μg/l, respectively, were also included. The bacteria were grown in 500-ml lots in 1.5-l Erlenmeyer flasks placed on a reciprocating shaker (100 oscillations/min) for 3 or 4 days at 30°. Cells deficient in cobalt were obtained only after growing the inocula through several (4) cultures of deficient media to deplete the micronutrient. They were collected in a continuous-flow Servall centrifuge at 35000 $\times$ *g* and washed in 0.85% (w/v) saline until all the nitrite was removed.

Nitrate reductase extracted from a cell paste by grinding with a similar weight of alumina powder (Alcoa-A 301), in a cold pestle and mortar, was taken up in 0.1 *M* phosphate buffer (pH 8). The homogenate was centrifuged at 20000 $\times$ *g* for 20 min and the supernatant solution used as a crude source of the enzyme. Considerably more nitrate reductase was extracted into the supernatant solution after pretreating the homogenate at 60° for 5 min, chilling quickly to 4° and storing overnight in a refrigerator. After this treatment, however, the enzyme was less stable. The enzyme was assayed anaerobically in small Thunberg tubes using reduced benzyl viologen as the hydrogen donor. The enzyme was also assayed aerobically when sodium succinate or DPNH was used as the hydrogen donor. Details of the nitrate reductase assays are given in Table I. Nitric oxide uptake was measured in a Warburg apparatus as described by FEWSON AND NICHOLAS[4]. Total nitrogen was determined by the micro-Kjeldahl method and protein by the Folin reaction[5].

The results in Table I show that a deficiency of cobalt diminished nitrate reductase activity when either sodium succinate, DPNH or BVH was the hydrogen donor. The uptake of nitric oxide was also reduced in cobalt-deficient cells. Nitrate reductase was extracted and purified from bacteria that had been grown in culture media containing 8 μg cobalt labelled with 4 μC ^{58}Co/l. The results of the fractionation shown in Table II indicate that the metal does not concentrate as the enzyme is

TABLE I

EFFECT OF COBALT DEFICIENCY ON NITRATE REDUCTASE ACTIVITY AND NITRIC OXIDE UPTAKE BY WHOLE CELLS OF *Rhizobium japonicum* (Strain 505)

Nitrate reductase was assayed as follows: (1) 10 μmoles sodium succinate, 80 μmoles phosphate buffer (pH 7.5), 10 μmoles KNO_3, 0.1 ml enzyme. Total volume, 1 ml. (2) 0.2 μmoles DPNH, 0.1 mg crystalline alcohol dehydrogenase, 0.1 ml 5% (v/v) ethanol, 40 μmoles phosphate buffer (pH 7.5), 10 μmoles KNO_3. Total volume, 1 ml. (3) The assay system in Thunberg tubes contained: 1.5 μmoles benzyl viologen, 30 μmoles phosphate buffer (pH 7.5), 3 mg palladized asbestos in the sidearm; and 10 μmoles KNO_3, 30 μmoles phosphate buffer (pH 7.5), 0.1 ml enzyme in the main well. Total volume, 1 ml. Air was rigorously removed from the tube and then pure hydrogen (passed through a Deoxo-catalytic deoxygenator) was introduced, when the benzyl viologen was reduced. The tube was evacuated again and the reduced benzyl viologen was drained into the reaction mixture (the pellet of asbestos retained in the sidearm). All assays were incubated for 10 min at 30°. Nitrite was determined in the reaction mixture after pretreatment with zinc acetate and alcohol as described elsewhere[6].

Expt.	*Co* (*µg/l*)	*Age of culture* (*days*)	*Yield dry weight* (*g/l*)	*Nitrate reductase* (*µmoles NO_2^-/mg N/h*)			*NO uptake* (*µmoles NO/mg N/h*)
				Hydrogen donor			
				1. Sodium succinate	*2. DPNH*	*3. BVH*	
1	0	3	0.47	1.6	3.8	34.0	0.10
	5	3	0.97	3.7	10.7	63.0	0.34
2	0	4	1.08			0.61	0.19
	5	4	1.72			7.3	0.28
3	0	4	0.54			0.48	0.23
	5	4	1.0			2.46	0.50

TABLE II

PURIFICATION OF NITRATE REDUCTASE FROM *Rhizobium japonicum* (Strain 505) AND THE COBALT CONTENT OF THE FRACTIONS

8 μg ^{58}Co (4 μC)/l were added to the basal culture solution and aliquots of the various enzyme fractions prepared from the bacteria assayed in a scintillation counter (thallium-activated sodium iodide crystal).

Fraction	*Procedure*	*Total protein (mg)*	*Total Co (mμg)*	*Total enzyme units (mμmoles NO_2^- per 10 min)*	*mμg Co per mg protein*	*Enzyme: Specific activity (mμmoles NO_2^- per 10 min/mg protein)*
1	Supernatant solution after centrifuging at 3000 × *g*	70	505	6840	7.2	97.7
2	Precipitate from 0–50% ammonium sulphate saturation of fraction (1) dissolved in 0.1 *M* phosphate buffer (pH 7.4)	31.2	38.1	4110	1.2	132
3	Calcium phosphate gel (200 mg) added to fraction (2) which is adjusted to pH 6 with acetic acid. Centrifuged at 3000 × *g* for 5 min. Gel eluted with 0.2 *M* phosphate buffer (pH 8.0). Enzyme activity of eluate measured	7.87	12.7	1850	1.6	236
4	Precipitate from 0–50% ammonium sulphate saturation of fraction (3) dissolved in 0.1 *M* phosphate buffer (pH 7.4)	5.77	5.25	1970	0.9	341
5	Precipitate from 50–90% ammonium sulphate saturation of fraction (1) dissolved in 0.1 *M* phosphate buffer (pH 7.4)	2.58	2.12	2010	8.0	770

purified. Further purification, however, will be necessary to establish unequivocally whether cobalt is a constituent of the enzyme. The addition of graded amounts of cobalt or vitamin B_{12}, to extracts prepared from cobalt-deficient cells or to purified fractions of the enzyme, did not activate nitrate reductase. The micronutrient was readily dialysed from the purified enzyme without an apparent loss of activity. These preliminary results suggest that cobalt is unlikely to be a functional constituent of nitrate reductase and that its effect on the enzyme may well be on its formation rather than on its action. This concept is further supported by the finding that the uptake of nitric oxide is also diminished by cobalt deficiency. Further work is in progress to elucidate the possible function of cobalt in the utilization of nitrate in the Rhizobia.

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Intracellular distribution of sialic acid and its relationship to membranes

In "sialopolymers" such as glycoproteins and glycolipids, sialic acid is almost always found terminally bound, and in certain tissues such as red blood cells it is considered to exist on the periphery of the cell. It was therefore of interest to determine the distribution of sialic acid in subcellular fractions of liver, especially with regard to the particulate membranes.

Excised livers from 250–300 g male, Wistar rats were perfused with cold 0.25 *M* sucrose and homogenized and fractionated according to the procedure of SCHNEIDER[1], except that the microsomes were obtained by centrifugation at 105 000 × *g* for 1 h in a Spinco preparative ultracentrifuge (Model L). Each particulate fraction was washed twice by resuspension and centrifugation. Preparations of mitochondrial