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A COBALT REQUIREMENT FOR NON-NODULATED LEGUMES AND FOR WHEAT

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Abstract—Cobalt deficiency was produced in non-nodulated subterranean clover, *Trifolium subterraneum*, grown with nitrate or urea nitrogen and in wheat, *Triticum durum*, supplied with nitrate nitrogen. Deficiency effects were also obtained in sterile cultures of *T. subterraneum* utilizing either cobaltous sulphate or cyanocobalamin as a source of the micro-nutrient. Cobamides were not detected in sterile *T. subterraneum* which rapidly degraded added ^{60}Co cyanocobalamin. It was shown, by gel filtration techniques, that over 90 per cent of the tracer is not associated with cobalamin nor inorganic cobalt. The separation of these cobalt complexes of low molecular weight from plant extracts is described.

INTRODUCTION

IT IS established that cobalt is essential for nitrogen fixation by free-living bacteria, blue-green algae and symbiotic systems.[1,2] The metal requirement is usually associated with the production of cobamide compounds by the bacteria which function in their metabolism.[3] Cobamide co-enzymes and methyl malonyl CoA isomerase which requires the co-enzyme have been found in the root nodules of legumes and non-legumes.[1,3]

Relatively little is known about the effect of the micronutrient on the non-nodulated plant. A cobalt deficiency has been reported in rubber and tomatoes[4] and in non-nodulated subterranean clover[5] but deficiency symptoms were not recorded. Traces of cobamide materials have been found in plant material grown under sterile conditions, by microbiological assay methods.[6] It is not known whether these compounds were derived from the seed or produced *de novo*. The expanding parts of plants accumulate cobalt and there is chromatographic evidence for a non-cobamide complex containing cobalt in root nodules.[7] The respiration rate of root tips from cobalt deficient *T. subterraneum* plants is reduced.[8]

In this paper, techniques are described for producing visible signs of cobalt deficiency in non-nodulated subterranean clover, *T. subterraneum* and in wheat *T. durum*. With the aid of cobalt labelled with ^{60}Co, complexes of the metal have been separated from plant extracts by filtration through Sephadex G-10 columns. It is shown that sterile plants degrade cobamide compounds readily and none was detected in extracts prepared from these.

[1] H. J. EVANS, S. A. RUSSELL and G. V. JOHNSON, In *Non-haem Iron Proteins; Role in Energy Metabolism* (Edited by A. S. PIETRO), p. 303. Antioch Press, Ohio (1965).
[2] D. J. D. NICHOLAS, M. KOBAYASHI and P. W. WILSON, *Proc. Natl Acad. Sci. U.S.* **48**, 1537 (1962).
[3] H. J. EVANS and M. KLIEWER, *Ann. N.Y. Acad. Sci.* **112**, 735 (1964).
[4] E. W. BOLLE-JONES and V. R. MALLIKARJUNESWARA, *J. Rubber Res. Inst. Malaya* **15**, 128 (1957).
[5] S. B. WILSON and E. G. HALLSWORTH, *Plant Soil* **22**, 260 (1965).
[6] L. FRIES, *Physiol. Plantarum* **15**, 566 (1962).
[7] S. B. WILSON and E. G. HALLSWORTH, *Plant Soil* **23**, 60 (1965).
[8] E. G. HALLSWORTH, S. B. WILSON and W. A. ADAMS, *Nature* **205**, 307 (1965).

RESULTS

Essentiality of Cobalt

The effects of a cobalt deficiency on non-nodulated subterraneum clover, *T. subterraneum*, and on wheat *T. durum* grown with nitrate nitrogen as the sole nitrogen source are illustrated in Fig. 1 and Table 1. The young leaves developed a chlorosis. Dry weight yields of tops and roots were considerably reduced in deficient culture as shown in Fig. 2A and B. A range of

TABLE 1. THE EFFECT OF ADDING COBALT AND CYANOCOBALAMIN TO DEFICIENT PLANTS OF *Trifolium subterraneum* (AGED 67 DAYS) GROWN UNDER STERILE CONDITIONS. PLANTS SUPPLIED WITH 15 MMOLES/L. NITRATE, 60 DAYS BEFORE HARVESTING

Treatment	No addition	Cyanocobalamin 0·1 μmoles/l.	Cobaltous sulphate 0·1 μmoles/l.
Mean dry weight(g)	0·24	0·36	0·34

Least difference for significance 0·08g, P=0·01.

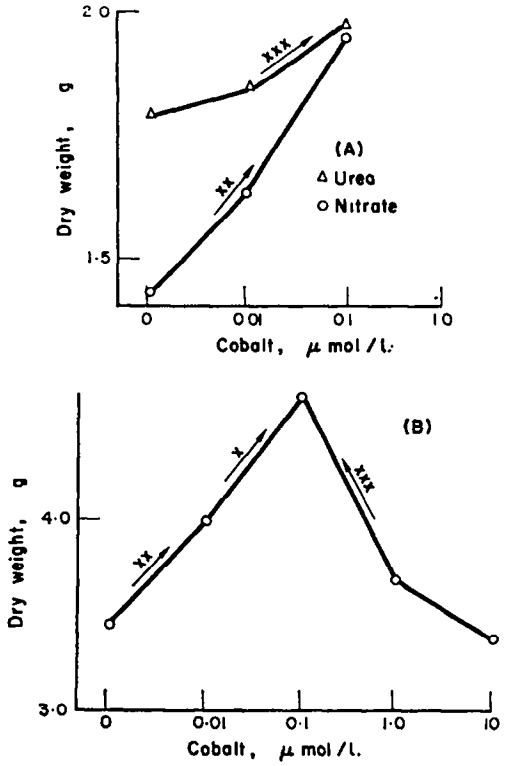

FIG. 2. THE EFFECTS OF COBALT ON THE DRY WEIGHTS OF PLANTS GROWING WITH 10 MMOLES/L. ADDED NITROGEN AS INDICATED.

(A) *Trifolium subterraneum* c.v. Mt. Barker. Non-nodulated plants supplied with nitrate or urea nitrogen. (B) *Triticum durum* c.v. Dural, supplied with nitrate only. Statistical significance: $\xrightarrow{x}$ P=0·05; $\xrightarrow{xx}$ P=0·01; $\xrightarrow{xxx}$ P=0·001.

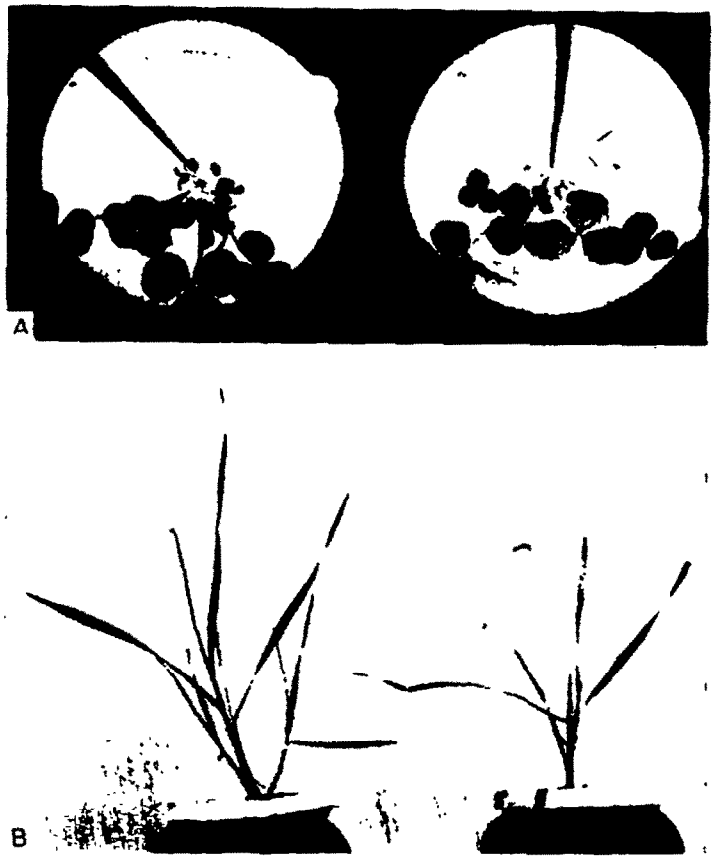

FIG. 1. THE EFFECT OF COBALT DEFICIENCY ON NON-NODULATED *Trifolium subterraneum* (AGE 39 DAYS) A, AND *Triticum durum* (AGE 28 DAYS) B.

Plants grown with 10 mmoles/l. nitrate as sole source of nitrogen. Left: plants grown with 0·1 μmoles cobalt/l.; Right: plants grown without added cobalt.

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mineral micronutrients included in the culture solution had no effect on the recovery of the plants. The response to cobalt was produced over the range 0·01 to 0·1 μmoles/l. In *Trifolium*, a cobalt requirement was also established when urea was substituted for nitrate nitrogen (Fig. 2A).

Since these plants were not grown under aseptic conditions, it is likely that microbial activity converted some of the cobalt to cobamide like materials and that these were taken up by the plants. Plants were then grown under sterile conditions with cobalt supplied as cyanocobalamin or cobaltous sulphate. The dry weights of the deficient plants were significantly lower than normal ones but there was no difference between cyanocobalamin or cobaltous sulphate-treatments.

Cobalt Complexes in Plants

Ethanol extracts of plants were supplemented with $CaCl_2$, cobaltous acetate and cyanocobalamin prior to passing through G-10 Sephadex columns. Ethanol was used to elute the various fractions. The results in Fig. 3A show the behaviour of $^{60}CoCl_2$ and ^{60}Co-cyanocobalamin alone and when added to extracts of tops of *Glycine max*. Unlabelled dicyanocobinamide was also eluted in the same volume as cyanocobalamin.

Extracts of all the plants examined whether sterile or non-sterile contained a large proportion of the label as complexes which were neither cobalamin nor inorganic cobalt by comparison with reference samples (Fig. 3B and 3C). Extracts of wheat grown with $^{60}CoCl_2$ did not contain any major amount of the tracer in the inorganic form (Fig. 3B,1) indicating that it had been metabolised. Cobalt complexes were not formed when $^{60}CoCl_2$ was added *in vitro* to these extracts. (Fig. 3B,1). The cobalt complexes occurred when either $^{60}CoCl_2$ or ^{60}Co cyanocobalamin was supplied *in vivo* to *T. subterraneum* or *Glycine max*. (Fig. 3C). It is noteworthy that the cobalamin fraction was not detected in extracts of sterile *T. subterraneum* supplied with $^{60}CoCl_2$ and was only a small proportion of the total activity in plant extracts when ^{60}Co cyanocobalamin was supplied to the plant (Fig. 3, C, 1 and 2). Samples of the various fractions collected from the Sephadex column and again passed separately through the same unit were all quantitatively eluted with the same volume of ethanol.

Production and Degradation of Cobamide Compounds in Trifolium

The results of the previous experiments suggest that no cobamides could be detected in sterile plants supplied with $^{60}CoCl_2$ and that ^{60}Co cyanocobalamin added to the plants *in vivo* was metabolised into unidentified cobalt containing complexes. The production and degradation of cobamides was examined further using the reversed isotope dilution analyses, bioassay and filtration through Sephadex. Ammonium formate (0·1 M) was added to the ethanol used for eluting the Sephadex columns to ensure that cobamide materials present would be readily removed from the columns.

The results of the reversed isotope dilution analysis given in Table 2 show that sterile plants of *T. subterraneum* grown for 31 days with $^{60}CoCl_2$ in the nutrient medium did not incorporate the tracer into free or bound cobalamins. When the plants were grown under non-sterile conditions however labelled cobalamins were formed presumably due to microbial activity. When ^{60}Co cyanocobalamin was added to the culture solutions of sterile plants, 30 days after germination, only 7 per cent of the labelled material in the ethanol extracts was cobamide after 5 days growth but none was detected in extracts of plants supplied with $^{60}CoCl_2$ instead of cyanocobalamin.

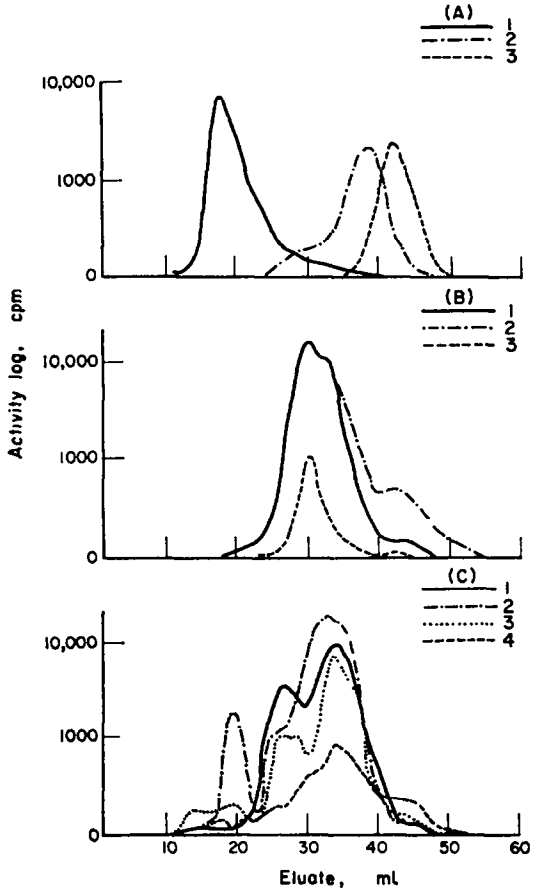

FIG. 3. CHROMATOGRAPHY OF 80 PER CENT BOILING ETHANOL EXTRACT OF PLANTS TREATED AS DESCRIBED IN THE TEXT AND PASSED THROUGH SEPHADEX G-10 (50 × 1 CM COLUMN). ELUTION WITH 65% V/V ETHANOL. CARRIER AMOUNTS OF COBALTOUS ACETATE, CYANOCOBALAMIN AND CALCIUM CHLORIDE WERE ADDED TO THE SAMPLES PRIOR TO PASSAGE THROUGH THE COLUMN (100 μG COBALTOUS ACETATE, 20 μG Co CYANOCOBALAMIN AND 20 MG $CaCl_2 2H_2O$). PLANTS GROWN WITH LABELLED MATERIAL 5 DAYS BEFORE HARVESTING.

(A) 1. ^{60}Co cyanocobalamin added to extracts of unlabelled *Glycine max* tops not previously grown with labelled materials or ^{60}Co cyanocobalamin alone. 2. $^{60}CoCl_2$ added to extracts of unlabelled *Glycine max* tops not previously grown with labelled materials. 3. $^{60}CoCl_2$ reference. (B) 1. $^{60}CoCl_2$ labelled non-sterile *Triticum durum* tops. Plants grown 31 days prior to the addition of labelled material. 2. $^{60}CoCl_2$ labelled non-sterile *T. durum* tops. Plants grown 31 days prior to the addition of labelled material. Additional $^{60}CoCl_2$ was added to the ethanol extract before passing through the column. This curve overlaps with the one obtained when $^{60}CoCl_2$ alone is passed through the column. 3. $^{60}CoCl_2$ labelled non-sterile *T. durum* roots. Plants grown 31 days prior to the addition of labelled material. (C) 1. $^{60}CoCl_2$ labelled sterile *T. subterraneum* plant. Plant grown 30 days prior to the addition of labelled material. 2. $^{60}CoCl_2$ labelled non-sterile *T. subterraneum* plant. Plant grown 45 days prior to the addition of labelled material. The peak at 30 ml has an elution volume between those for cobalamin (18 ml) and inorganic cobalt (40 ml) which have molecular weights of 1200 and 60 respectively. 3. ^{60}Co-cyanocobalamin labelled sterile *T. subterraneum* plants. Plants grown for 31 days prior to the addition of labelled material to the culture solution. 4. $^{60}CoCl_2$ labelled non-sterile *Glycine max* tops. Nodulated plant grown for 60 days prior to the addition of labelled material to the culture solution.

TABLE 2. THE INCORPORATION OF $^{60}CoCl_2$ AND ^{60}Co-CYANOCOBALAMIN INTO *Trifolium subterraneum*

Labelled compound in agar nutrient medium	Age of plant (days) At isotope addition	Age of plant (days) At harvest	Extracts of sterile (S) or non-sterile (NS) plants	^{60}Co C.P.M.
$^{60}CoCl_2$	3	34	(NS) 0·1 M acetate buffer pH 4·8+0·01% KCN	2610
$^{60}CoCl_2$	3	34	(S) 0·1 M acetate buffer pH 4·8+0·01% KCN	0
$^{60}CoCl_2$	3	34	(S) Papain digest of plant residues after 0·1 M acetate buffer pH 4·8+0·01% KCN extraction	0
$^{60}CoCl_2$	30	35	(S) 80% v/v boiling ethanol	0
^{60}Co-cyanocobalamin	30	35	(S) 80% v/v boiling ethanol	120

When *T. subterraneum* was grown under aseptic conditions in culture media treated with activated charcoal to remove corrin materials, cobamide compounds were not detected in extracts of these plants by the *Escherichia coli* C181 tube assay. About 90 per cent of ^{60}Co cyanocobalamin added to a plant extract was recovered by the charcoal technique.

No labelled cobamide compounds were detected on Sephadex G-10 columns from ethanol extracts of sterile *T. subterraneum* plants (Fig. 4B,1). The first and last labelled fraction (15 and 40 ml respectively) eluted from Sephadex columns with an ethanol-formate mixture, were identified as cobamide and cobaltous ions respectively by comparison with authentic markers (Fig. 4A). It is noteworthy that dicyanocobinamide is eluted from the column in the same fraction as cobamide. In extracts of non-sterile *Glycine max* supplied with ^{60}Co cyanocobalamin into the base of the stem, about 4 per cent of the activity was associated with cyanocobalamin (Fig. 4B,2).

The amounts of ^{60}Co cyanocobalamin fell rapidly in extracts of sterile *T. subterraneum* collected at intervals after adding the labelled material to the culture solution (Fig. 4C). The addition of cyanide to the buffer used to elute the columns had no effect on the activity of the cobamide fraction, thus indicating that all the cobamide materials had been eluted (Fig. 4D,1).

Hydrolysis of extracts of plants given ^{60}Co cyanocobalamin with 0·1 N formic acid prior to passage through the column converted the fraction collected at 20 ml to one at 40 ml. The latter peak coincides with that of inorganic cobalt. At the end of the experiment the agar medium contained cyanocobalamin only (Fig. 4D,3).

DISCUSSION

The data presented herein leave little doubt that cobalt is required for the growth of non-nodulated subterranean clover, *T. subterraneum* and wheat, *T. durum*. It is noteworthy that previous attempts to establish a cobalt deficiency in non-nodulated plants were made with a large seeded species having substantial reserves of the micronutrient.[9] In our experiments however, seed reserves were considerably reduced by excising embryos from small seeds of plants taken from cobalt deficient areas. Although in previous work rigorous methods were used to remove cobalt from the culture solution, as was done in this study, the control of the atmospheric contamination was not attempted.[10] In our experiments air entering the growth chamber was rigorously purified by passage through special filters.

[9] S. AHMED and H. J. EVANS, *Proc. Natl Acad. Sci. U.S.* 47, 24 (1961).
[10] A. J. ABBOTT and E. J. HEWITT, *Ann. Rep. Long Ashton Res. Sta., Bristol Univ.* p. 145 (1965).

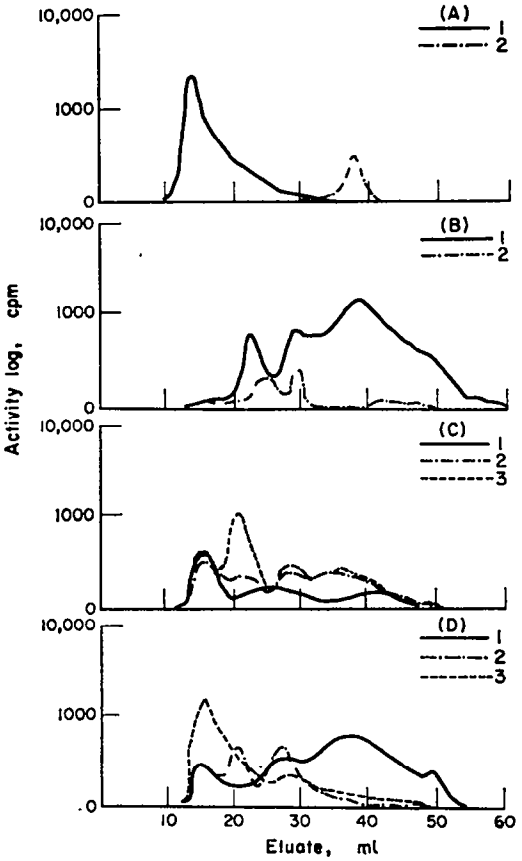

FIG. 4. CHROMATOGRAPHY OF 80 PER CENT BOILING ETHANOL EXTRACTS OF PLANTS TREATED AS DESCRIBED IN THE TEXT AND PASSED THROUGH SEPHADEX G-10 (50 × 1 CM COLUMNS). ELUTION WITH 65% V/V ETHANOL CONTAINING 0·1 M AMMONIUM FORMATE. CARRIER AMOUNTS OF COBALTOUS ACETATE AND CYANOCOBALAMIN (10 μG COBALTOUS ACETATE AND 20 μG Co CYANOCOBALAMIN) WERE ADDED TO THE EXTRACTS PRIOR TO PASSAGE THROUGH THE COLUMNS.

(A) 1. ^{60}Co cyanocobalamin added to extracts of *Glycine max* tops not previously grown with labelled materials. 2. $^{60}CoCl_2$ added to extracts of *Glycine max* tops not previously grown with labelled materials. (B) 1. Sterile *T. subterraneum* tops. Plants supplied with $^{60}CoCl_2$ after 30 days growth and harvested 5 days later. 2. Non-sterile *Glycine max* tops. Plants supplied with ^{60}Co cyanocobalamin after 60 days growth and harvested 5 days later. (C) 1. Sterile *T. subterraneum* plants. Plants supplied with ^{60}Co cyanocobalamin after 30 days growth and harvested at once. 2. Sterile *T. subterraneum* plants. Plants supplied with ^{60}Co cyanocobalamin after 30 days growth and harvested 1 day later. 3. Sterile *T. subterraneum* plants. Plants supplied with ^{60}Co cyanocobalamin after 30 days growth and harvested 5 days later. (D) 1. Sterile *T. subterraneum* plants. Plants supplied with ^{60}Co cyanocobalamin after 30 days growth and harvested 5 days later. Extract treated with 0·1 N formic acid for 15 min and neutralised before passage through the column. 2. Sterile *T. subterraneum* plants. Plants supplied with ^{60}Co cyanocobalamin after 30 days growth and harvested 5 days later. Eluting solution 65% v/v ethanol containing 0·1 M ammonium formate and 0·01% KCN. 3. Residual radioactive material in nutrient agar after removal of sterile *T. subterraneum*. Plants supplied with ^{60}Co cyanocobalamin after 30 days growth and harvested 5 days later.

The fact that in our experiments cobamide compounds were not detected in sterile plants of *T. subterraneum* is correlated with the rapid degradation of cyanocobalamin introduced into these plants. Thus cobamide compounds are readily broken down by plant enzymes. This observation is in agreement with the similar growth response obtained with either cobalt or cyanocobalamin when added to deficient plants in sterile conditions. These results differ from those reported by Fries[6] who claims that in *Pisum sativum* cobamide compounds are produced. It is not clear from this work whether the large reserves which were laid down under non-sterile conditions might have contained cobamide as contaminants derived from microflora.

It is clear that cobalt containing complexes other than cobamide materials are formed in *T. subterraneum*, *Glycine max* and *T. durum*. Since these compounds from different plants, separate in a similar way on Sephadex columns, it is of interest to speculate that they may be common to higher plants much as cobamide compounds are associated with micro-organisms. Further work will be necessary to establish the composition, function and distribution of these compounds in nature.

EXPERIMENTAL

Culture of Plants

Non-sterile plants were grown in solution culture. Cobalt was removed from A.R. nutrient salts by the α-nitroso-β-naphthol method.[4] Ferric citrate was prepared from $FeCl_3$ the latter purified by the anion exchange method described previously.[7,11] "Specpure" mineral micronutrients (Johnson Mathey Ltd. London) were used without further purification. The composition of the culture solution is given in Table 3. Purified water prepared by distilling rain water in a pyrex glass still and then passing it through an 80 × 10 cm column of mixed bed resin (2 parts by weight Dowex 21K and 1 part Dowex 50). The conductivity of the water was < 0·1 μmhos. The pH of the culture solution was adjusted by adding 1 mequiv/l. of $NaHCO_3$, the latter treated with dithizone to remove cobalt. Excess dithizone was eliminated by two treatments with activated charcoal.

TABLE 3. COMPOSITION OF THE NUTRIENT SOLUTIONS

Reagent	Concentration Element/L
KCl	2·0 mmoles K
$CaCl_2$ (dried)	1·6 mmoles Ca
$MgSO_4.7H_2O$	0·75 mmoles Mg
	0·75 mmoles S
$Na_2HPO_4.2H_2O$	0·32 mmoles P
Ferric citrate	0·075 mmoles Fe
$MnSO_4.4H_2O$	0·0075 mmoles Mn
$CuSO_4.5H_2O$	1·0 μmoles Cu
$NaNO_3$	10 mmoles N
or	
Urea	10 mmoles N
$ZnSO_4.7H_2O$	1·0 μmoles Zn
H_3BO_4	30 μmoles B
$(NH_4)_6Mo_7O_{24}.4H_2O$	0·20 μmoles Mo
$Al_2(SO_4)_3.18H_2O$	0·33 μmoles Al
$NiSO_4.6H_2O$	0·10 μmoles Ni
$GaCl_2$	0·066 μmoles Ga
$CoSO_4.7H_2O$	Variable Co

[11] K. A. KRAUS and F. NELSON, In *Proc. Int. Congr. Peaceful Uses of Atomic Energy*, Vol. 8, p. 837. Geneva (1955).

The plants were housed in a growth cabinet illuminated by banks of 12 × 400 W mercury lamps and 6 × 40 W fluorescent lamps separated from the growing compartment by a glass screen. The light intensity at plant level was 50 g cal/cm²/day, the day length 15 hr and the day and night temperature 20° and 15° respectively. Circulating air in the growth cabinet was filtered, through "Dycon" filters (Westinghouse Roseberry Pty. Ltd., Roseberry, N.S.W.) to remove dust particles. All metal surfaces were painted with clear varnish and trays of purified water placed on the floor to maintain the humidity. *T. subterraneum* plants were grown in 300 ml polystyrene containers blackened on the outside with "Bituros" solution (Wailes Dove Ltd., Hebburn, Durham) and *T. durum* plants in 1 l. high density polythene containers. The plants were supported in a small hole drilled in the white clip on lids which were blackened on the underside with "Bituros" solution.

All apparatus was cleaned with chromic acid other than the high density polythene containers which were treated with aqua-regia and then conc HCl (1 + 1) followed by at least five washes with purified water.

Air supplies for aerated cultures were passed first through a 60 cm column of charcoal granules and then through N H_2SO_4 and finally filtered through a 60 cm column of acid washed glass wool. In non-static cultures the culture solution was circulated and aerated by the use of compressed air from a reservoir through a series of 500 ml high density polythene culture vessels linked by polythene tubes. This system which prevented damage to root systems by air bubbles, was easy to clean and regulate and enabled all the culture solutions in one treatment to be charged without handling either the vessels or the plants. *T. subterraneum* c.v. Mt Barker were obtained from field plots where cobalt deficiency had occurred in nodulated plants but cobalt deficiency could also be obtained with commercially produced seed if the cotyledons were excised at the time of emergence of the unifoliate leaf. *T. durum* c.v. Dural, seeds were obtained from plots at the Waite Institute and the embryos were excised when the coleoptiles were 4–5 cm high.

Seeds of uniform weight were washed with 0·02% Nonidet P40 detergent (Shell Chemical Co.) five times with purified water before sterilizing with 3% v/v H_2O_2 for 10 min[12] and washed a further five times with purified water before germination at 20° on acid washed filter paper. Seedlings selected by uniformity were placed in the culture vessels when the radicle of *Trifolium* or the coleoptile of *Triticum* was 1 cm long and a further rigorous selection for uniformity was made 21 days after germination. Experimental treatments were started when the plants were 21 days old using 0·01 μmoles/l. of cobalt and raised in tenfold steps (logarithmic scale) at weekly intervals until the final concentrations were reached (see Results).

Sterile plants were grown from seeds surface sterilized with 95% v/v redistilled ethanol for 3 min followed by 0·1% w/v mercuric chloride for 4 min and washed four times with sterile glass distilled water. Culture media and vessels were sterilized by autoclaving for 15 min at 15 lb/in².

Seeds employed in the deficiency experiments were individually germinated on fibre glass mesh supported in 6 × ½ in. pyrex test tubes capped with inverted specimen tubes. The tubes were filled to the level of the glass mesh with purified culture solution. At the time of emergence of the unifoliate leaf (7–10 days) aliquots of the culture solutions from each tube were plated on yeast extract, peptone, mineral salts medium to check sterility prior to transplanting. The cotyledons were removed asceptically before transfer to larger containers.

The sterile plants were cultured in 2-l. pyrex beakers covered with an inverted pyrex petri dish. The annulus was sealed with cotton wool and the plants supported at culture solution level with fibre glass mesh resting on an inverted high density polythene jar which was drilled to allow the root to pass through. Each plant was supplied with 1200 ml of the original purified culture containing nitrate nitrogen and supplemented with 0·32 mmoles/l. phosphorus, 5 mmoles/l. nitrate nitrogen. Experimental treatments were started at the time of transfer to the large containers at 0·01 μmoles/l. of cobalt or the equivalent amount of cyanocobalamin (0·1 μmoles/l.) and raised three weeks later to 0·1 μmoles/l. cobalt by adding sterile cobaltous sulphate or cyanocobalamin. An aliquot from each culture vessel was further tested for sterility by plating before harvesting.

Non-deficient plants were sterilized as described previously and germinated directly on the yeast extract peptone mineral salts medium. Individual sterile seedlings were transferred after three days to 8 × 1 in. pyrex test tubes containing 25 ml unpurified nutrient solution supplemented with 0·1 μmoles/l. cobaltous sulphate and solidified with 0·7% agar. The tubes were plugged with non-absorbent cotton wool. The semi-solid medium thus allowed easy removal of the plant. Samples of the medium in each tube were tested for sterility before harvest.

Dry Weight

The plants were dried at 80° for 48 hr in an aerated oven followed by cooling in a desiccator.

Radioactive Isotope Techniques

^{60}Co was obtained as the chloride specific activity 7·6 mC/mg Co, from the Australian Atomic Energy Commission, Lucas Heights, Sydney, N.S.W. ^{60}Co labelled cyanocobalamin "Rubratope 60" specific activity 1·09 μc/μg was a gift from Dr. D. Perlman, Squibb Institute for Medical Research, New Brunswick,

[12] K. T. WIERINGA, In *Nutrition of the Legumes* (Edited by E. G. HALLSWORTH), p. 256. Butterworths, London (1958).

New Jersey, U.S.A. Benzyl alcohol present in the Rubratope as a preservative was removed by drying the material in a stream of oxygen-free nitrogen at 50°.

^{60}Co was radioassayed in a 2 in. well type thallium activated sodium iodide crystal using an "Echo" 610 scaler and automatic sample changer.

Separation of Non-cobamide Complexes containing Coba

Plants grown in unpurified nutrient solution were supplied with 2 μC ^{60}Co per plant using cotton wicks to transfer the isotope from small vials into the base of the stem and the plants harvested 5 days later. Negligible amounts of the tracer were detected in the culture solution.

After harvest the plant parts were plunged into boiling absolute ethanol which was maintained at boiling point for 2 min. After cooling and decanting the alcohol, the tissue was re-extracted twice with boiling 80% v/v ethanol. The combined extracts were dried in a rotary evaporator with *n*-propanol to prevent the accumulation of a water rich mixture. The extracts of the tops were treated with light petroleum to remove chlorophyl and the residues finally extracted with absolute methanol. The latter solution contained 90 per cent of the radioactivity. Recovery from the columns was 100 per cent of the tracer added and at least 90 per cent of the tracer in the plant was recovered in the ethanol extracts.

The methanol solutions were passed through Sephadex G-10 columns (50 × 1 cm) eluting with 65% ethanol; inactive cobaltous acetate, cyanocobalamin and calcium chloride were added to each sample prior to placing on the column. Satisfactory separation was not achieved in the absence of either ethanol or calcium chloride.

Determination of Cobalamins Produced by Plants

Three methods were used to determine cobalamins (a) bioassay, (b) reversed isotope dilution assay and (c) Sephadex filtration.

(a) Sterile plants used for bioassay were grown in culture media containing 0·1 μmoles/l. cobalt. Cobamides were removed from the solution by treating it with activated charcoal. The charcoal was purified by boiling in several batches of AR grade HCl (1 + 1) washed three times with purified water and then with hot 10% w/v phenol and hot 65% acetone. This treatment would remove any cobamide that might be present in the culture solution. Cobamides were extracted from the plants by boiling the tissue for 30 min in 0·1M sodium acetate buffer (pH 4·8) containing 0·01% KCN.[13] After homogenising this material for 5 min in a top drive macerator it was centrifuged at 30,000 *g* for 15 min. The supernatant fraction was collected, the residue re-extracted with purified water and these washings added to the original extract which was then treated with charcoal at pH 3, 4, 5, 6, 7 and 8 respectively.[6] The charcoal fractions were bulked and treated with 5% w/v phenol[14] and purified water and the cobamides were eluted with several batches of boiling 65% v/v acetone. The combined acetone fractions were dried in a rotary evaporator at 50°. Purified water was then added and the material dried again, this process was repeated until all the phenol was removed. The residue was dissolved in purified water and transferred into sample tubes. It was then dried in a stream of oxygen free nitrogen at 50°. The cobamide contents of these samples were assayed by the *Escherischia coli* C181 tube assay. The residue from the acetate buffer extract was resuspended in acetate buffer (pH 7·0) and digested asceptically with papain (50 mg enzyme g residue) for 48 hr at 37° in the presence of 0·02 M cysteine hydrochloride. The usual checks were made to confirm that conditions were sterile. The cobamide materials were then determined by the experimental procedure described previously for the soluble fractions.

(b) For the reversed isotope dilution assay, sterile plants were grown with $^{60}CoCl_2$ or ^{60}Co cyanocobalamin (0·2 μC/plant). When $^{60}CoCl_2$ was used, cobalt was omitted from the culture medium because there was sufficient of the metal in the unpurified medium to support normal growth. The cobamides were extracted from the plants by the acetate buffer (pH 4·8) method and by papain digestion or by boiling 80% v/v ethanol.

^{60}Co in the plant extracts as cobalamins were recovered in the presence of carrier amounts of inactive cobalt and cyanocobalamin (600 μg and 500 μg, respectively) by extracting with benzyl alcohol.[15] After extracting the dicyanocobalamin with benzyl alcohol the organic phase was washed several times with 20% w/v Na_2SO_4 containing 1% KCN (pH 11·5). When the dicyanocobalamin complex was transferred back into the water phase it was washed several times with benzyl alcohol: $CHCl_3$ mixture (1 : 1). The radioactivity present in the washings was checked to ensure that no more of the tracer was present before proceeding with the purification. The spectrum of the purified dicyanocobamide (5 cm cells SP700 spectrophotometer) was found to be identical with a sample of crystalline cyanocobalamin tested with excess KCN. Dicyanocobalamin was determined from the optical density measured at 582 nm and from the radioactivity of the purified complex corrected for losses during purification.

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(c) Cobamides were separated from ethanol extracts using G-10 Sephadex columns (50 × 1 cm) eluted with 65% v/v ethanol containing 0·1 M ammonium formate. In some experiments, 0·01% w/v KCN was included in the eluting solution. Carrier amounts of inactive cobaltous acetate and cyanocobalamin were added to each sample before passing through the column.

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