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THE ESSENTIALITY OF TRACE ELEMENTS FOR MICRO-ORGANISMS AND THE MICROBIOLOGICAL DETERMINATION OF THESE ELEMENTS

E. G. MULDER

(Agricultural Experiment Station and Institute for Soil Research T.N.O., Groningen, Holland)

The essentiality of trace elements for micro-organisms, particularly for bacteria, is less well established than that for higher green plants. This may be ascribed partly to the fact that most micro-organisms require for their normal development in addition to the mineral nutrients one or more organic compounds. Owing to this the quantity of cell material formed per unit of added nutrients is considerably higher in green plants than in micro-organisms. Since the amounts of required trace elements are closely related to the amount of cell substances synthesized by the organism, it will be understood that the effect of trace element impurities in the nutrients is of more importance in micro-organisms than is the case with higher green plants. This effect may be accentuated by the difficulty or purifying organic compounds from mineral elements.

A further complication which may be encountered in studying trace element requirements of bacteria is the change in metabolic processes under the influence of certain deficiencies without affecting materially the breakdown of organic compounds and the rate of growth of the organism. Iron-deficient cultures of *Clostridium welchii* were found by Pappenheimer and Shaskan (41) to convert glucose mainly to lactic acid. Addition of iron shifted the fermentation to yield acetate, butyrate, ethyl alcohol, CO_2 and H_2. Similar results were obtained by Simon (45) with *Clostridium acetobutylicum* which in carbon monoxide treated cultures yielded lactic acid from glucose. Without this treatment normal butyl fermentation occurred. The accumulation of fumaric acid in zinc-deficient culture solutions of *Rhizopus* [Foster and Waksman, (16)] and of citric acid in zinc-deficient cultures of some strains of *Aspergillus niger* may be explained in a similar way.

The picture becomes still more complicated if alternative non metal requiring pathways exist which come into action when the medium becomes deficient in the particular metal. Examples of the occurrence of such a mechanism in *Torulopsis*,

Candida and *Clostridium acetobutylicum* have been recorded by Knight (25). In iron-deficient culture solutions riboflavin production was increased. Apparently under such conditions the organisms survive by using riboflavin or flavoprotein instead of metal-containing hydrogen carrier systems.

The ability of other elements to substitute for a certain trace element apparently is of more importance in bacteria than in green plants. It is a well-known fact that a number of enzymatic reactions *in vitro* may be catalyzed by different trace elements. In many cases in which manganese plays a rôle as a cofactor this element may be replaced by magnesium, cobalt, nickel or some other metallic ions. It is assumed that in reactions of this type the trace elements are functioning as a « cementing agent » between the protein and the prosthetic group (25) or between the enzyme and the substrate (47). Some examples of enzymatic reactions of this type are: phosphoglucomutase, which catalyzes the interconversion of glucose-1-phosphate and glucose-6-phosphate, and enolase which catalyzes the formation of phospho-enolpyruvate from D-2-phosphoglycerate. Mg^{++}, Mn^{++}, or Zn^{++} are active as a cofactor. Isocitric dehydrogenase which catalyzes the transformation of isocitric acid to α-ketoglutaric acid, requires either Mn^{++} or Mg^{++} [von Euler *et al.*, (13), Adler *et al.*, (2), Ochoa, (40)]. Oxalacetic carboxylase which is responsible for the splitting of oxalacetic acid in pyruvate and carbon dioxide was found to be activated by Mn^{++}. Ca^{++}, Co^{++}, Cd^{++} and Zn^{++} could replace the Mn^{++}, acting only slightly less efficiently, while Pb^{++}, Ni^{++}, Fe^{++}, Mg^{++}, Cu^{++} and Ba^{++}, in the absence of Mn, proved to have some effect (48).

So far little is known of the substitution of trace elements in organisms living under natural conditions. In those cases where rate of growth or appearance of the organism are being used as the criteria for trace element deficiencies, it is improbable that such a substitution will be observed, because an element would have to substitute for all of the functions of the essential elements in the organism concerned. In those cases, however, where the effect of a certain element on a particular reaction has to be studied, it is highly probable that results similar to those found in enzymatical studies will be obtained. The data which can be found in the literature on replacement of certain elements by a number of others may be considered as evidence of the latter statement. However, the results obtained by various authors generally are rather conflicting [see the review of Perlman, (42)]. Partly this may be ascribed to the fact that widely differing amounts of trace elements have been used by various authors. As a result of this, inhibition and contamination effects sometimes may have played a rôle. A further difficulty is that different fungus strains may behave differently as to their response to trace elements.

So far the following trace elements have been found to be essential for microorganisms: Fe, Mn, Cu, Zn and Mo. This is true of *Aspergillus niger* with which most of the experiments have been carried out [Steinberg (49), (50)]. Growth rate and sporulation often have been used as a criterion for a trace element deficiency of

the fungus in these investigations. By using the proper purification methods Donald, Passey and Swaby (12) succeeded in limiting the yield of mycelium of *Aspergillus niger* grown in metal-deficient cultures to the following values (expressed as % of yield of complete medium): Fe: 5, Mn: 36, Cu: 33, Zn: 1, Mo: 11.

Although other fungi presumably respond in a similar way to these trace elements there is far less complete evidence available in the literature as to their essentialitiy. The same is true of yeasts and particularly of bacteria. Iron and manganese have been shown to be essential for a number of bacteria. As regards zinc, copper and molybdenum the results obtained so far are insufficient to decide whether these elements are of general importance for bacteria. For certain bacteria their essentiality has been demonstrated as will be seen below.

In addition to the above-mentioned elements, B, Co and presumably Ga have to be considered as essential trace elements for certain types of micro-organisms. The rôle of Ni, Cd, Pb and of a number of other elements in the normal metabolism of micro-organisms is unknown. These elements may be substituted for some essential trace elements in a number of enzymatical reactions *in vitro* as well as *in vivo*.

Iron.

Since iron is a constituent of a number of enzymes e.g. the cytochromes, cytochrome oxidase, catalase, peroxidase, the former of which regulate the energy transfer in nearly every cell, it is quite understandable that omitting of this element is disastrous for practically every living organism. This is demonstrated by numerous investigations with fungi, yeasts and bacteria. In addition to the above-mentioned iron is also functioning in a number of other enzymes (hydrogenase, formic dehydrogenase, formic hydrogenylase). Waring and Werkman showed that when insufficient amounts of Fe were present, the cytochrome system of *Aerobacter indologenes* was satisfied first. Only iron in excess of the amount required for the cytochromes was available to the other enzymes (54).

The importance of iron for the formation of acetate, butyrate etc. by *Clostridium welchii* has been reported earlier in this report.

In experiments with *Aspergillus niger* by the author it was found that when nitrate was the sole nitrogen source the fungus needed more iron than was the case with ammonium nitrogen. Apparently an iron-containing enzyme functions directly or indirectly in nitrate reduction.

Manganese.

The essentiality of manganese for fungi has been shown by many authors. In the case of *Aspergillus niger* no spores develop when inadequate amounts of Mn are present. No continuous mat is formed on the medium and the hyphae have a ball-

like appearance. According to Tomlinson *et al.* (52) manganese in sub-optimal concentrations favours the formation of citric acid by *Aspergillus niger*.

In bacteria the essentiality of Mn has been shown for a group of lactic acid bacteria.

Copper.

The importance of copper for a number of fungi and for some bacteria has been studied by the author (27), (28), (29). In a copper-deficient medium *Aspergillus niger* developed a poor mycelium without sporulation. Supplied with 0.4 μg of Cu per 40 ml of nutrient solution formation of dry matter was nearly optimal but the spores had a yellow-brown colour instead of the normal black. Approximately 2 μg of Cu were needed for the formation of black spores. In these tests copper could not be replaced by the following elements: Li, Ba, Cd, Pb, Sn, Ag, Co, Ti, Va, Al, B, J, Mo. When these elements were added in a 40-times higher concentration than copper, no effect on the activity of copper was found, except with cadmium. The latter was found to antagonize the activity of copper as far as coloration of spores concerns (table 1).

TABLE 1

EFFECT OF CADMIUM AND COPPER SULPHATES ON SPORE COLOUR OF *ASPERGILLUS NIGER*

3 $CdSO_4 \cdot 8\ H_2O$ μg	Cu, μg per 40 ml of nutrient solution (as sulphate)			
	0	2	6	20
0	light-yellow	black	black	black
25	light-yellow	black	black	black
50	light-yellow	brown-black	black	black
100	light-yellow	brown-black	black	black
200	no spores	brown-gray	brown-black	black
500	no spores	yellow	brown-black	brown-black
1000	no spores	yellow	brown	brown-black
2000	no spores	yellow	yellow-brown	brown

With increasing amounts of added copper increased acidity of the nutrient solution was obtained (27), (28). These results were confirmed by Tomlinson *et al.* (52), (53) who found four times as much citric acid formed in the case of 4 μg of Cu added per 100 ml of nutrient solution than without added copper. Weight of mycelium was nearly 40 per cent higher in the former case.

In addition to *Aspergillus niger*, *A. glaucus*, *A. flavus* and *Penicillium glaucum*

were found to require copper for their normal development. Weight of mycelium as well as sporulation and colour of spores were clearly affected (27), (28).

No effect of copper on nitrogen fixation and growth of *Azotobacter chroococcum* was found but the black pigment of the colonies which developed in the presence of added copper was not formed when no copper was added.

The oxidation of manganous carbonate to MnO_2 by a fungus growing on a sodium acetate containing nutrient medium was considerably enhanced by addition of a trace of copper sulphate (27), (28).

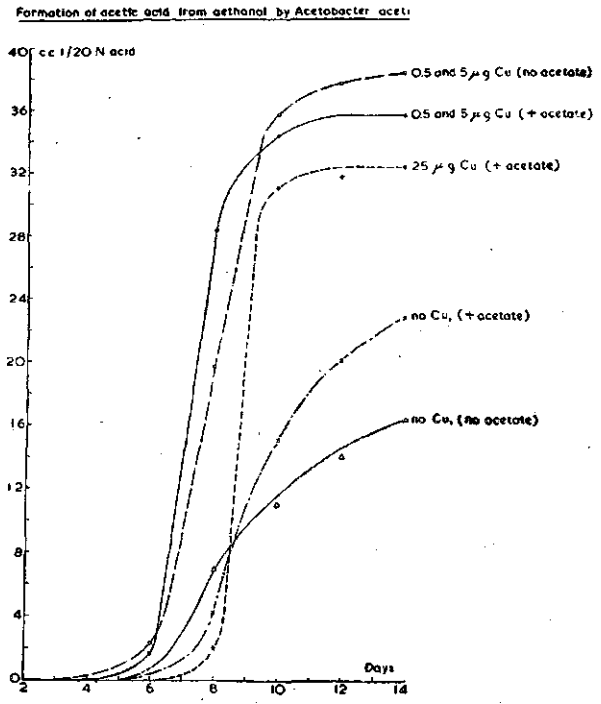

Fig. 1

Acetobacter aceti was found to require traces of copper for the oxidation of ethyl alcohol to acetic acid. This was true in the presence as well as in the absence of added acetate (fig. 1).

Copper has been found as a constituent of tyrosinase and other phenolases and of ascorbic acid oxidase. Although it should therefore be highly probable that the black pigment in the spores of *Aspergillus niger* as well as that of ageing cultures of *Azotobacter chroococcum* depends on the formation of melanin from phenolic compounds by copper-containing phenolases, no such enzymes could be detected in these micro-organisms.

Rate of respiration in *Aspergillus niger* as measured by oxygen uptake was found to be considerably higher in copper-deficient mycelia than in mycelia supplied with traces of copper.

Zinc.

The importance of zinc for fungi is a well-known fact. In the absence of this element *Aspergillus niger* was found to produce less than 1 per cent of the dry weight obtained at an optimal zinc level [Steinberg, (51)]. Sub-optimal concentrations were found by Foster and Waksman (16) to be highly favourable for formation of fumaric acid from glucose by *Rhizopus nigricans*. Although the breakdown of glucose in the zinc-deficient cultures was not reduced, the yield of mycelium was only 40 per cent of that of mycelia grown at an optimal concentration of zinc. This was due to the poor efficiency of carbon utilization for cell synthesis.

Formation of penicillin by *Penicillium notatum* was found by Foster *et al.* to be higher in nutrient solutions with optimal zinc supply than in zinc-deficient solutions (17). Apparently this phenomenon is related to the effect of zinc on pH, since pH of the nutrient medium is important for penicillin production. Nason, Kaplan and Colowich found that the enzyme which catalyzes synthesis of tryptophan from serine and indole is completely absent in zinc-deficient *Neurospora* (36). Alcohol dehydrogenase activity was also low in the latter case. Phosphatase activity was found by Robinson (see 56) to be much increased in zinc-deficient tomato plants. It was suggested by Wood (56) that failure of tryptophan formation which is often observed in zinc-deficient organisms could be attributed to destruction of pyridoxal phosphate as a result of the increased phosphatase activity. Pyridoxal phosphate plays a part as a coenzyme in the synthesis of tryptophan from serine and indole.

Boron.

Evidence concerning the importance of boron for microbiological processes was presented by Brenchley and Thornton (10) more than 25 years ago in connection with nitrogen fixation by beans (*Vicia faba*). These results have been confirmed by the author in experiments with pea plants (31). No development of nodules on the roots of these plants was observed in nutrient solutions to which no boron was added, notwithstanding the culture solution as well as the root system had been inoculated with an effective strain of *Rhizobium*. Due to the lack of nodules the nitrogen supply of the boron-deficient plants became inadequate (table 2); light green leaves appeared and the plants died from nitrogen starvation. In the presence of a trace of boron many nodules were seen on the roots and the leaves had a dark green appearance.

In this experiment no symptoms of boron deficiency were seen in the leaves and the buds of the plants. The secondary roots of the B-deficient plants were very short as compared with the long roots of the B-supplied plants. Since the activity of boron

in higher plants generally is confined to the growing tissues, it is highly probable that the lack of nodules was due to a reduced growing capacity of the root tissue rather than to a reduced activity of the bacteria.

In a further experiment, inoculated pea plants were grown in nutrient solutions with increasing amounts of boron. In order to find out to what extent an application of combined nitrogen may decrease the boron requirement of the plants, one set of plants was supplied with combined nitrogen. The results of this experiment showed that the boron requirement of the green tops of pea plants is considerably higher than that of the nodules. Once the nodules have been formed, nitrogen fixation may take place irrespective of the occurrence of B-deficiency symptoms in the tops.

TABLE 2

EFFECT OF BORON ON THE NITROGEN FIXATION BY PEA PLANTS

H_3BO_3 mg per pot	Yield, dry weight, g		N in total plants mg (*)
	Tops	Roots	
0	1.10	0.93	40.7
0.5	3.10	1.04	110.8

(*) The nutrient solution was initially supplied with a very small amount of combined nitrogen.

Jordan and Anderson (24) have studied the effect of boron on nitrogen fixation by *Azotobacter chroococcum*. Although they claim a relatively large increase in fixed nitrogen, their absolute nitrogen figures are low. Recently Gerretsen has obtained more convincing results as to the essentiality of boron for *Azotobacter chroococcum* (19).

Koffler *et al.* (26) found that borax increased the penicillin yields of *Penicillium chrysogenum*.

Eyster (15) found boron an essential element for an alga viz. *Nostoc muscorum*. The reduction in cell count for an eight-week growth period was 39 per cent for illuminated cultures. The boron-deficient cells were completely white. Addition of boron corrected the chlorosis. The optimum B-concentration appeared to be about 0.1 p.p.m.

Molybdenum.

A considerable amount of work on molybdenum has been carried out recently in the author's laboratory (30), (33), (34). In agreement with earlier investigations

by Steinberg (50) it was shown that *Aspergillus niger* requires traces of this element when cultivated in a medium supplied with nitrate nitrogen. Vanadium which partly can replace molybdenum in the fixation of gaseous nitrogen by *Azotobacter* had no effect on the growth of *Aspergillus niger* when added to a molybdenum-deficient nutrient solution.

The amounts of molybdenum required for a normal growth of the fungus are approximately a hundred times smaller than those of required copper. When ammonium sulphate had been added to the molybdenum-deficient medium, growth was much improved. It was shown that the difference in molybdenum requirement of nitrate and ammonium cultures was not a result of a difference in molybdenum impurities in these nitrogenous compounds. It can be concluded from these results that in a nutrient solution with nitrate as the nitrogen source *Aspergillus niger* requires a higher molybdenum supply than in a medium with ammonium nitrogen.

EFFECT OF MOLYBDENUM ON DENITRIFICATION TABLE 3

Bact. strain	$Na_2MoO_4.2\ H_2O$ per bottle, mg	After 10 days incubation at 25°C	
		NO_3-N disappeared (*), mg	NO_2-N per bottle, mg
W 1	0	1.4	1.7
W 1	5	9.3	8.5
S 1	0	4.5	0.0
S 1	5	13.1	0.0
S 2	0	0.0	0.0
S 2	5	10.2	8.1
W 2	0	0.0	0.0
W 2	5	8.0	7.4

(*) Initial nitrate concentration: 13.1 mg N per bottle (50 cc).

Nicholas (37) estimated the nitrate content of *Aspergillus niger* grown in the absence of added molybdenum. A value of 1050 µg of nitrate-N per gram of fresh weight of fungus was found, whereas no nitrate was detected in a mycelium supplied with 0.02 µg of Mo per culture. This author found also a marked reduction in amino acid content in Mo-deficient *Aspergillus niger* as estimated by paper chromatography.

In agreement with the results obtained with *Aspergillus niger* the author found molybdenum to be essential for denitrification (30). In these experiments four strains of denitrifying bacteria were incubated in stoppered bottles filled with a glucose-containing nutrient medium. Part of the bottles had received 5 µg of $Na_2MoO_4 \cdot 2$

H_2O. The results of this experiment clearly show that molybdenum is indispensable for denitrification (table 3).

In experiments with higher green plants (tomato, barley) similar results were obtained (30). Plants deficient in molybdenum were found to be unable to assimilate added nitrate. The latter accumulated in the leaves to a high degree but no increase in organic nitrogen compounds was found. When ammonium sulphate was added as the nitrogen source a ready assimilation was observed in molybdenum-deficient tomato. Nitrate accumulation in molybdenum-deficient green plants has also been recorded by Hewitt and Jones (20) and by Wilson and Waring (55).

TABLE 4

EFFECT OF MOLYBDENUM AND NITROGEN NUTRITION OF CAULIFLOWER AND TOMATO PLANTS ON NITRATE REDUCING CAPACITY OF LEAF AND STEM TISSUES (*)

Plant tissue	µg $NaNO_2$ formed in ½ h at 37°C.		Treatment of plants
	per 1 g dry weight	per 1 mg protein	
Cauliflower, leaf	42	0.3	O Mo + nitrate
Cauliflower, leaf	293	1.6	low » + »
Cauliflower, leaf	790	3.0	normal » + »
Cauliflower, leaf	363	1.7	normal » O »
Cauliflower, stem	121	1.6	O » + »
Cauliflower, stem	206	3.0	low » + »
Cauliflower, stem	329	5.9	normal » + »
Cauliflower, stem	178	2.5	normal » O »
Tomato, leaf	34	0.2	O » + »
Tomato, leaf	40	0.2	low » + »
Tomato, leaf	453	2.0	normal » + »
Tomato, leaf	37	0.3	normal » O »
Tomato, stem	101	1.5	O » + »
Tomato, stem	209	3.4	low » + »
Tomato, stem	387	6.1	normal » + »
Tomato, stem	158	3.2	normal » O »

(*) 1 g finely cut, fresh plant tissue, 5 cc 0.06 M phosphate buffer (pH 7.1), 1 cc 0.1 M malic acid, 1 cc 01 M KNO_3, 2 cc H_2O (anaerobically, Thunberg tubes).

The results of these experiments with micro-organisms as well as those with green plants indicate that molybdenum is intimately related to nitrate reduction. It is unknown, however, whether molybdenum participates in some enzymatical reaction governing nitrate reduction (« nitrate reductase ») or that it is functioning in

an energy-supplying reaction which indirectly may affect nitrate reduction. The fact that molybdenum-deficient cauliflower plants supplied with ammonium nitrogen, although considerably darker green than nitrate plants, made only slightly better growth than the latter, indicates that an inadequate nitrate reduction is not the main cause of the poor growth of molybdenum-deficient cauliflower.

In experiments with cauliflower and tomato plants carried out in the author's laboratory it was found that nitrate reduction in finely cut leaf and stem tissues, suspended in a malic acid solution, proceeded much more readily in plants supplied with molybdenum than in molybdenum-deficient plants (see table 4). It is not yet decided whether these results demonstrate the essentiality of molybdenum for a « nitrate reductase » or for malic dehydrogenase. According to Nason and Evans (14), (35) nitrate reduction in *Neurospora* as well as in green plants depends on the presence of a flavin nucleotide containing enzyme (« nitrate reductase ») which mediates electron transport between nitrate and reduced triphosphopyridine nucleotide (TPNH) or reduced diphosphopyridine nucleotide (DPNH):

$$NO_3^- + TPNH + H^+ \rightarrow NO_2^- + TPN + H_2O.$$

DPNH can be readily supplied by malic dehydrogenase which reduces DPN by oxidation of malic acid. Although these authors were unable to demonstrate a rôle of molybdenum in « nitrate reductase » of soy bean plants, according to a later communication by Nicholas, Nason and McElroy (39), « nitrate reductase » of *Aspergillus niger* and of *Neurospora* would be a molybdenum-containing enzyme. Richert and Westerfeld (43) demonstrated the presence of molybdenum in xanthine oxidase.

The fact that malic dehydrogenase was found in the author's laboratory to be more active in cauliflower and bean plants supplied with molybdenum than in molybdenum-deficient plants, while lower E_h values were found in the former plants than in the latter, indicates a possible function of Mo in malic dehydrogenase and possibly in other dehydrogenases.

A third possibility is that the poor activity of the nitrate-reducing system in molybdenum-deficient plants is due to a poor formation of the protein part of the enzyme (cf. the values for nitrogen-deficient tomato and cauliflower leaves in table 4).

Catalase activity was found to be very low in molybdenum-deficient bean, cauliflower and tomato leaves as compared with normal leaves. The same is true of nodules of molybdenum-deficient clover and lucerne plants.

An important observation has been made recently by Spencer in Wood's laboratory in respect of the function of molybdenum in living organisms (56). In agreement with observations by Courtois and Bossard (11), Bossard (9) and Rothstein and Meier (44) that molybdate may inhibit phosphatase activity, it was found that in molybdenum-deficient tomato leaves phosphatase activity was more pronounced than in plants supplied with molybdenum. As a result of this, inorganic phosphate in

molybdenum-deficient plants was found to be higher than in plants supplied with molybdenum whereas some organic phosphates were lower. It has been suggested that in molybdenum-deficient plants hexose phosphates and ATP are present in low concentrations owing to phosphatase activity and that consequently the glycolytic and respiratory cycles provide H for nitrate reduction and ATP for organic nitrogen synthesis at a reduced rate. These experiments have been repeated in the author's laboratory. We were unable to confirm Spencer's results. Molybdenum-deficient tomato and cauliflower plants were found to be somewhat lower in phosphatase activity than plants which had been supplied with molybdenum. The inhibiting effect of molybdate in concentrations according to Rothstein and Meier on phosphatase activity in macerates of tomato leaves could be confirmed.

TABLE 5

EFFECT OF MOLYBDENUM ON NITROGEN FIXATION BY WHITE CLOVER, GROWN IN DIFFERENT SOILS

Yield, g per pot		N, mg per pot	
No added Mo	0.5 mg Na_2MoO_4. 2 H_2O per pot	No added Mo	0.5 mg Na_2MoO_4 per pot
1.74	1.96	44.4	63.4
0.74	1.94	18.0	58.3
1.47	2.28	50.8	76.9
2.71	3.81	79.3	121.6
1.99	3.70	63.0	126.4
2.52	3.17	85.3	102.3
3.12	2.88	101.0	91.9
2.46	3.40	79.6	114.2
2.74	3.73	88.8	122.0
3.50	2.96	126.7	95.3
2.87	3.70	93.0	120.0
0.28	1.26	6.6	40.4
1.64	2.35	50.9	74.8
1.47	2.89	44.5	92.4
1.15	3.43	29.0	114.0
1.54	3.65	40.0	109.9
0.28	0.89	8.2	31.1
2.06	2.52	61.1	76.2
0.52	1.86	13.0	58.4
0.68	2.34	14.5	68.8
1.33	2.36	42.1	77.0

In addition to the effect of molybdenum in nitrate reduction, this element plays an important rôle in the fixation of gaseous nitrogen by free-living micro-organisms

as well as by symbiotic nitrogen fixers. This was shown for *Azotobacter chroococcum* and for nitrogen-fixing algae by Bortels (6), (7), (8), for *Clostridium butyricum* and *Azotobacter indicum* by Jensen and Spencer (21) and Jensen (23).

When *Azotobacter* is grown with nitrate nitrogen the amount of molybdenum required for normal development is considerably lower than is the case with gaseous nitrogen. Supplied with ammonium nitrogen molybdenum apparently is not required (30).

The importance of molybdenum for nitrogen fixation by leguminous plants growing in acid ironstone-containing soils was shown approximately ten years ago by Australian workers (3), (4).

TABLE 6

EFFECT OF Mo ON YIELD OF CLOVERS AND LUCERNE (FIELD EXPERIMENT)

$Na_2MoO_4 . 2 H_2O$ kg per ha	White clover		Red clover		Lucerne	
	Air-dried matter, q/ha	N in dry tops, %	Air-dried matter, q/ha	N in dry tops, %	Air-dried matter, q/ha	N in dry tops, %
1st cutting						
0	17.9	2.99	9.3	2.56	6.5	2.23
2	34.2	3.15	27.0	2.75	17.7	2.73
4	30.4	3.01	26.5	2.83	26.3	3.47
2nd cutting						
0	26.0	3.43	33.7	2.53	6.0	2.53
2	33.5	3.54	38.7	3.13	23.2	3.60
4	33.2	3.37	38.6	2.96	20.6	2.84

Some years later the author demonstrated the essentiality of molybdenum for the fixation of gaseous nitrogen by pea plants growing in nutrient solutions. In the absence of added molybdenum many nodules developed but these were unable to fix nitrogen so that the plants died at an early stage owing to nitrogen deficiency (30).

A poor fixation of elementary nitrogen by leguminous plants owing to molybdenum-deficiency was observed in low moor peat soils rich in ironstone. Such soils frequently may be found in the Netherlands along brooklets. Table 5 gives the results of a pot experiment with white clover. It will be seen that in the majority of the soils tested the nitrogen fixation by the nodules of the white clover was considerably improved by traces of molybdenum. When nitrate nitrogen had been added to such plants no response to molybdenum was observed. This shows that the molybdenum supply of the plants for fixation of elementary nitrogen has to be considerably higher than that for nitrate reduction. This behaviour is in agreement with that of *Azotobacter chroococcum* as has been stated earlier.

In field experiment with white clover, red clover and lucerne, it was found that the latter requires the highest doses of molybdenum (table 6). Although this may depend on a higher requirement of molybdenum [see also Jensen (22)] it is probable that a difference in uptake of soil molybdenum exists between clover and lucerne. This may be concluded from the fact that in the second cutting the untreated clover plants had succeeded in absorbing soil molybdenum in amounts nearly sufficient for normal nitrogen fixation. The lucerne plants, however, had been unable to reach this level. The difference in molybdenum-absorbing capacity between clover and lucerne was further shown when the leguminous crops were ploughed up and cauliflower was cultivated. This plant is very sensitive to molybdenum-deficiency and may be used as an indicator plant. It appeared that after red clover symptoms of molybdenum deficiency were less severe than after lucerne.

Cobalt.

Cobalt is the center of a complex in vitamin B 12. This vitamin is required by a number of bacteria. Many micro-organisms synthesize it. This means that cobalt apparently is an essential element for these organisms. The author does not know any investigation in which the growth of a micro-organism was markedly affected by cobalt deficiency.

Estimation of trace elements by microbiological methods.

During the last 15 years microbiological assay methods have been worked out for the estimation of a number of trace elements in various materials. These methods are used particularly for ascertaining the level of plant-available trace elements in soil. It is believed that the availability of a certain element in a microbiological test is in accordance with its availability to the roots of higher green plants. Generally such a parallelism exists but in some cases the results of microbiological methods may agree less favourably with those of pot and field experiments with green plants. This anomaly presumably depends on the fact that the mechanism of uptake of trace elements by plant roots is different from that by micro-organisms. As a result of these differences the interacting effect of other elements on the uptake of a certain trace element may differ widely in higher plants and in micro-organisms. Some examples of these interactions will be given below.

Most workers have used *Aspergillus niger* for the estimation of plant-available trace elements in soil. Donald, Passey and Swaby (12) have made tests with 9 fungi, 1 alga, 1 yeast and 6 bacteria and found *A. niger* the most convenient organism. It grows rapidly in a nutrient medium containing glucose or saccharose as the only organic compound and it produces a fair amount of mycelium which under optimal nutritional conditions is densely covered with black spores. The latter is important

in those cases in which density of sporulation and spore colour are used as an index for availability of trace elements (copper and sometimes magnesium).

Although iron can be estimated by the *Aspergillus* method, according to Donald *et al.* (12) it is impossible to detect in this way iron deficiency in neutral or alkaline soils known to be deficient in iron, owing to the presence of oxides of iron, unavailable to plants. In the *Aspergillus* test these iron oxides can be used as an iron source. Similar difficulties were encountered by these authors in the estimation of plant-available manganese. Manganese deficiency of higher plants often depends on the occurrence of manganese in the form of higher oxides of manganese. These oxides are unavailable to the green plant but according to Donald, Passey and Swaby they can be used by *Aspergillus niger* as a manganese source.

For the determination of small amounts of manganese in various materials Bently, Snell and Phillips (5) used *Lactobacillus arabinosus*. Acid production from glucose as measured by titration was used as a measure for manganese.

The *Aspergillus niger* test has been used by the author for estimating plant-available copper, molybdenum and magnesium in soil and in plant material. Although magnesium is not considered to be a trace element, the results obtained with this element agree closely with those obtained with micronutrients so that their reporting seems worth-wile.

The bioassay for copper depends on the fact that within the range from 0-2 µg of added Cu per 40 ml of purified nutrient solution the spore colour of *Aspergillus niger* changes from white (sporulation considerably reduced or absent) through light yellow, brown-yellow, brown and black-brown to black. By adding 1 g of air-dried soil to a purified nutrient solution, inoculating with a spore suspension of the fungus and comparing the spore colour after 4 or 5 days incubation with the colour of a set of standard cultures, estimation of the amount of available copper in the soil is possible. In these experiments a good agreement between the results of pot and field experiments and those of the *Aspergillus* test was found (28), (29), (32).

In general it was found that soils on which the plants showed severe symptoms of copper deficiency contained 0.4 µg or less available copper per gram of air dry soil. Amounts from 0.6-1.5 µg Cu were found for soils on which the symptoms of Cu-deficiency were less severe, whereas normal soils had copper values higher than 2.0 µg per 1 g. These results are in agreement with those found by Acock (1) and Donald *et al.* (12) for Australian soils and by Nicholas and Fielding (38) for soils in Great Britain.

The amounts of molybdenum which can be estimated by the *Aspergillus* method are a hundred times lower than those of copper. This results from the fact that the fungus requires extremely small amounts of this element for its normal growth.

For the estimation of available molybdenum 100 mg of air dry soil is added to 50 ml of nutrient solution of pH 2. This pH value was found by Nicholas (37) to give the best agreement with visual symptoms and molybdenum status of cauli-

flower plants. After 5 days incubation at 30° C the mycelia are dried, weighed and compared with the mycelium weights of a standard series. Table 7 gives the results of an experiment in which the results of the *Aspergillus* method are compared with those of pot experiments with white clover and cauliflower as the test plants. It will be seen that soils, on which the higher plants respond to molybdenum dressings, in

TABLE 7

RESPONSE OF WHITE CLOVER AND CAULIFLOWER PLANTS TO MOLYBDENUM DRESSINGS IN RELATION TO *ASPERGILLUS*-AVAILABLE MOLYBDENUM OF VARIOUS SOILS

Soil type	pH	Available Mo (*Asp. niger*) p.p.m.	White clover, mg N in crop per pot		Cauliflower, response to added Mo
			O Mo	+Mo	
low moor peat (Fe) (*)	5.5	0.003	43.9	110.8	—
sandy (Fe)	6.1	0.005	44.0	92.4	strong
low moor peat (Fe)	6.0	0.006	6.6	40.4	—
sandy (Fe)	5.5	0.007	29.0	114.0	—
sandy (Fe)	4.6	0.007	—	—	—
sandy (Fe)	5.7	0.035	40.0	109.9	—
sandy	5.3	0.044	18.0	58.3	—
sandy (Fe)	5.4	0.044	14.5	68.8	strong
sandy	5.6	0.052	50.8	76.9	slight
sandy (Fe)	5.8	0.063	63.0	126.4	strong
sandy loam	—	0.100	no response		no response
sandy, normal arable soil	5.6	0.125	—	—	—
low moor peat (Fe)	5.8	0.153	51.6	158.7	strong
sandy loam, fertile garden soil	7.0	>0.4	—	—	—
sandy (Fe), Tasmania (Kinburn)	6.2	0.017	110.5	382.0	—
sandy (Fe), South Australia (Houghton)	5.4	0.002	93.4	170.0	—
peaty, Exp. field 119	4.1	0.008	no response (**)		strong
sandy, Exp. field 10	4.0	0.046	»	»	slight
peaty, Exp. field 24	4.0	0.093	»	»	no response
peaty, Exp. field 13	3.9	0.112	»	»	moderate
peaty, Exp. field 836	3.5	>0.2	»	»	strong
sandy, Exp. field 263	3.7	>0.2	»	»	no symptoms
sandy, Exp. field 837	3.7	>0.2	»	»	no symptoms
low moor peat field 200	4.0	>0.2	»	»	strong

(*) (Fe)=rich in ironstone.

(**) Nodulation was attained by mixing the acid soils with a small amount of slightly acid molybdenum-deficient soil which had been heavily inoculated with Rhizobium.

general have molybdenum values between 0.003 and 0.05 p.p.m. This situation was found in a group of slightly acid soils, mostly rich in ironstone. Normal soils are considerably higher in available molybdenum.

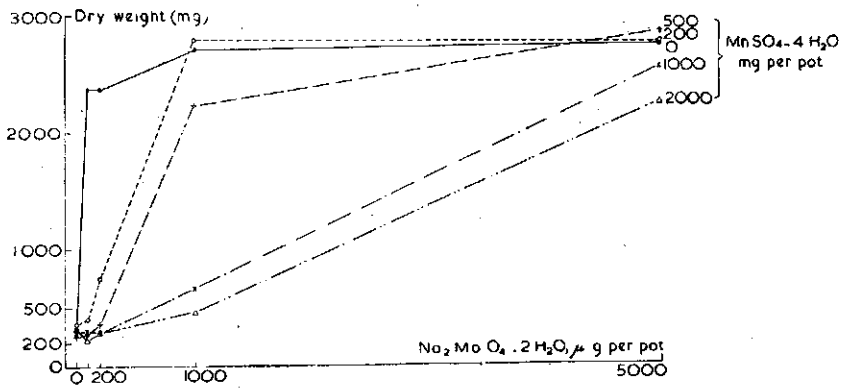

Fig. 2 - Effect of different amounts of sodium molybdate and manganese sulphate on yield of cauliflower grown in a molybdenum-deficient soil.

The results of a second group of soils with very low pH values on which cauliflower plants but not white clover responded to molybdenum treatment are quite

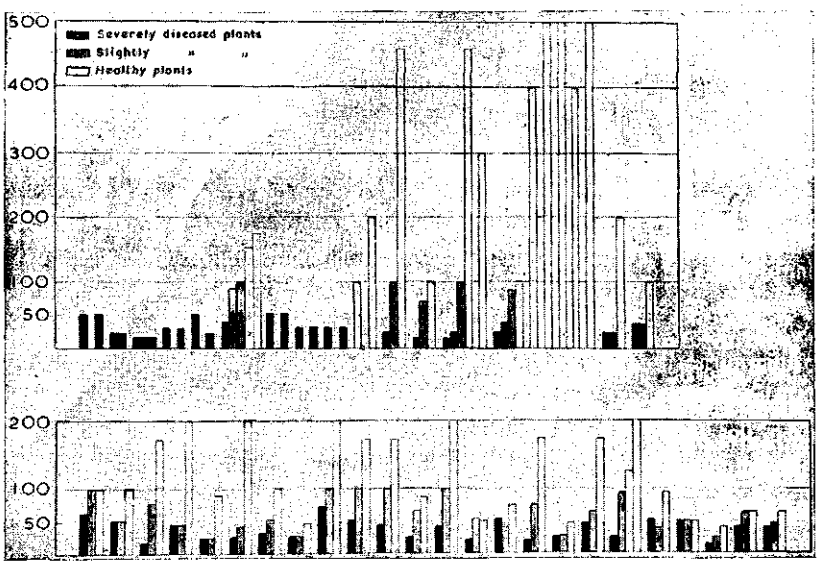

Fig. 3 - Available magnesium (µg per 3 g of air dry soil) as determined by *Aspergillus niger*. Samples from areas with normal and magnesium-deficient plants from a number of fields.

different from those of the first group. Soils of this type were relatively rich in available molybdenum (see table 7). The reason of this disagreement between the *Aspergillus niger* test and the cauliflower test with very acid soils presumably has to be sought in the different effect of interacting ions on molybdenum uptake of these two organisms. It was found that manganese sulphate antagonizes the uptake of molybdenum by cauliflower plants (see fig. 2). This effect was found to be partly due to the Mn^{++}, partly to SO_4^{--}. The uptake of molybdenum by *Aspergillus*

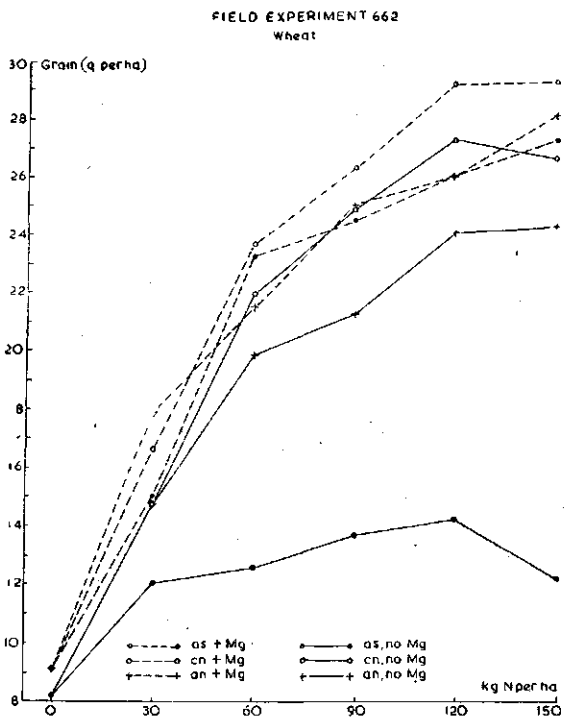

Fig. 4 - Wheat grown in a sandy soil (organic matter 6%, pH 5.3), relatively poor in available magnesium, with different amounts of ammonium sulphate (as), calcium nitrate (cn) and ammonium nitrate limestone (an).

niger, however, is not affected by manganese sulphate. Since it is a well-known fact that acid soils contain large amounts of soluble manganese, it is highly probable that the latter is responsible for the disagreement between microbiological test and growth of higher plants. Apparently the *Aspergillus* test gives a more reliable picture of the molybdenum status of soils than does the cauliflower test.

A similar result was obtained in testing the magnesium status of soils (46). In general a good agreement was found between appearance and growth of higher

plants and available magnesium as determined with *Aspergillus niger* (see fig. 3). The uptake of magnesium by higher plants was found to be strongly affected by the form in which the nitrogen is supplied (see fig. 5). NH_4- compounds antagonize the uptake of Mg, whereas nitrates have a favourable effect. K-ions likewise strongly antagonize the uptake of Mg-ions by higher plants. The uptake of magnesium by *Aspergillus* however, is neither affected by the form in which the nitrogen is supplied nor by an excessive amount of potassium (fig. 5). This demonstrates that the *Aspergillus* method is a more accurate test for ascertaining available magnesium than is the test with higher plants.

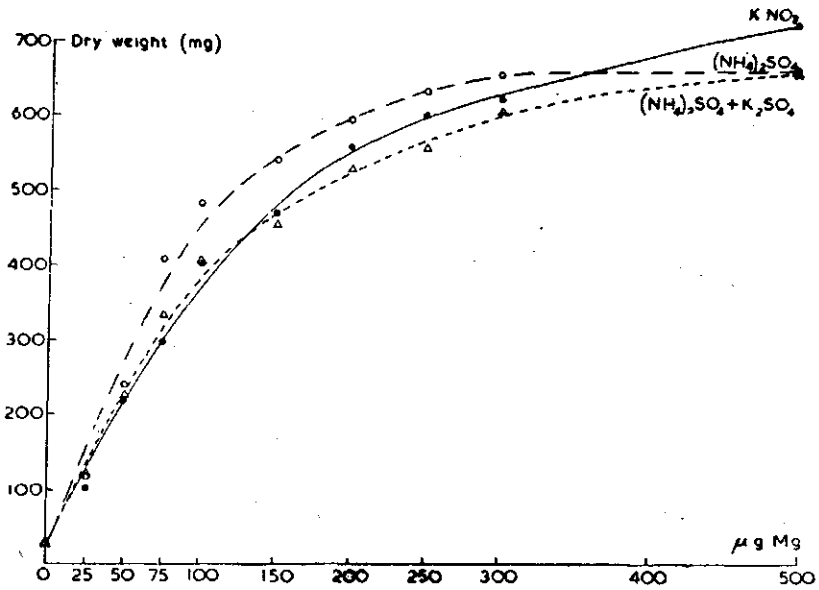

Fig. 5 - *Aspergillus niger* in nutrient solutions with increasing amounts of magnesium sulphate. Nitrogen in the form of KNO_3 and $(NH_4)_2\ SO_4$. In the latter case K as in KNO3 and double this amount respectively.

A bioassay method for zinc using *Aspergillus niger* as the test organism has been described by Gerretsen (18), Nicholas and Fielding (38), and Donald, Passey and Swaby (12). The latter found 0-0.5 µg Zn per g of very deficient soil, 0.6-2.0 µg in deficient soil and 2.2-12.0 µg in slightly deficient soil. Apparently the results of the biological zinc determination are in agreement with those of the test with higher plants.

Summarizing the results obtained by various authors with the *Aspergillus niger* test it can be said that the extreme sensitiveness of the organism enables a quick and fairly accurate estimation of small amounts of trace elements. This is of particular

importance in those cases in which plant-available trace elements in soil have to be determined. Although with proper extractants similar results may be obtained, the chemical methods will be found to be much more laborious and time consuming than the biological tests.

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