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**SUPPLEMENTARY ISSUE**

Comprising papers read at the Symposium on Trace Elements in Soils, Plants and Animals organized by the Agricultural Group and the Bristol Section of the Society of Chemical Industry, held at Bristol, 10-12 April, 1957.

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## IRON CHELATES IN PLANT NUTRITION\*

By C. BOULD

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The rôle of iron chelates in nutrient solutions and their use in pot and field experiments for controlling heavy metal- and lime-induced chlorosis in plants is described. Their stability in aqueous solutions and in soils, and their absorption, translocation and metabolism by plants are discussed. Reference is made to recent work on chelating agents which indicates that they may act as plant growth promoters.

**Culture solutions**

Iron complexes have been used in culture work for many years. According to Stewart & Leonard,<sup>1</sup> Guillon used iron citrate, iron malonate and iron tannate as sources of iron in 1895, but the first comprehensive studies on iron complexes were made by Gile & Carrero.<sup>2</sup> Reed & Haas<sup>3</sup> confirmed the effectiveness of iron citrate and iron tartrate in maintaining iron in solution; in addition they found iron salicylate to be satisfactory. Heck & Bailey,<sup>4</sup> however, showed that some strong chelating agents such as carbamate, nitroso-R-salt and quinalizarin, when added to nutrient solutions, induced iron deficiency in plants.

In 1950, Hutner *et al.*<sup>5</sup> used the iron chelate of ethylenediaminetetra-acetic acid (Fe-EDTA) in growth studies with algae, but Jacobson<sup>6</sup> was the first to carry out extensive culture experiments on higher plants with this iron chelate. He found that Fe-EDTA (1:1 molar ratio) was completely stable for 3 months when added to nutrient solutions of pH 6.0 at the level of 5 p.p.m. Fe, and stored in the dark; at pH 7.0, 18% Fe was precipitated and at pH 8.0 approximately 30% Fe was lost. Even at pH 9.0, 10% Fe remained in solution. From his studies on tomato, sunflower, corn and barley, Jacobson concluded that 5–10 p.p.m. Fe, as Fe-EDTA, was the optimum level for these crops. Injury to tomato and corn plants occurred with levels exceeding 50 p.p.m. of Fe.

Stewart & Leonard<sup>1</sup> found 5 p.p.m. of Fe, as Fe-EDTA, suitable for the growth of citrus in sand culture, but Woltz<sup>7</sup> noted some toxicity symptoms in gladioli when 2 p.p.m. of Fe were used.

De Kock<sup>8</sup> showed that some chelating agents were more effective than ferric chloride in alleviating iron chlorosis in mustard seedlings grown in nutrient solution at pH 7.8 in the presence of bicarbonate ions. When these compounds were used as a source of iron at rates equivalent to 1 p.p.m. of Fe the intensity of chlorosis in the plants followed the order: ferric chloride = Fe-EDTA > ferric Versenol (Fe-HEEDTA) > ferric Versente-diol (Fe-HEEDDA).

De Kock & Strmecki<sup>9</sup> called attention to the growth-promoting effect of lignite, which they attributed to its property of chelating iron and other metals; experiments with tomato seedlings at Long Ashton have indicated that iron lignin sulphonate (a by-product of the paper industry) can supply the iron requirements of these plants in culture solution.

The evidence so far available indicates that the iron chelates of polyaminopolycarboxylic acids (i.e., Fe-EDTA, etc.) are the most effective carriers of iron for nutrient culture work especially at pH values above 7.0.

**Soils**

The first successful use of iron chelates under field conditions was made by Stewart & Leonard.<sup>10</sup> They found that as little as 10–20 g. of Fe, as Fe-EDTA, per tree gave a satisfactory control of chlorosis in citrus trees on acid soils (pH 4.0): this form of chlorosis was shown by Reuther & Smith<sup>11</sup> to be copper-induced. Much larger amounts of iron, ranging from 100–300 g. of Fe per tree, were found by Leonard & Stewart<sup>12</sup> to be necessary for the control of lime-induced chlorosis, rates which the authors considered to be uneconomic. This, and other unfavourable results on alkaline soils, led to a search for other agents whose iron chelates were more stable

\* Read at Symposium on Trace Elements in Soils, Plants and Animals, organized by the Agriculture Group and Bristol Section, 10–12 April, 1957

than Fe-EDTA. Chaberek & Bersworth<sup>13</sup> described two such chelating agents, namely *NN'*-dihydroxyethylethylenediaminediacetic acid (HEEDDA or Versene-diol) and *N*-hydroxy-ethylethylenediaminetriacetic acid (HEEDTA or Versenol). The 1:1 ferric chelates of these two acids are more stable than Fe-EDTA in alkaline aqueous solutions.

Wallace *et al.*<sup>14</sup> found that the iron chelates of HEEDDA, HEEDTA and diethylenetriamine-penta-acetic acid (DTPA or Chel-330 Fe) were more effective than Fe-EDTA in correcting lime-induced chlorosis in ornamental trees, but Leonard & Stewart<sup>15</sup> obtained conflicting results with these iron chelates on citrus. On one soil, with pH 8.1 and a calcium carbonate content varying from 15.5% in the surface soil to 31% in the sub-soil, trees receiving from 75–100 g. of Fe, as Fe-HEEDTA, per tree made a complete recovery whereas on two other sites, with similar soils, treatments were ineffective.

Working with severely chlorotic pears, on a highly calcareous soil, Bould<sup>16</sup> obtained a complete seasonal control of chlorosis from relatively small dressings of Fe-EDTA (32 g. of Fe per tree) and Fe-DTPA (23 g. of Fe per tree) by thoroughly watering-in the chelates after soil application. Later he showed<sup>17</sup> that further economy could be made by sub-soil injection of aqueous solutions into the rooting zone of the tree.

Recently, Wallace *et al.*<sup>18</sup> and Holmes & Brown<sup>19</sup> tested the iron chelates of two new agents, namely *cyclohexane-1:2*-diaminetetra-acetic acid (CDTA) and an aromatic polyamino-poly-carboxylic acid (formula not available, referred to as Chel-138), both of which proved more effective in controlling chlorosis in alkaline calcareous soils than any other iron chelate tested; Chel-138 even mobilized iron from calcareous soils. This chelate and one described by Gasser & Müller<sup>20</sup> under the code-number RA-157 appear to be the most promising iron chelates for use on alkaline soils.

Although the iron chelates of natural products have not been used to any great extent, Wallace & Ashcroft<sup>21</sup> claimed some success with the iron chelate of ammonium lignin sulphonate (waste product from the paper industry) in controlling chlorosis in soya-bean. However, they found that very large applications were necessary (2000 lb. per acre as compared with 6–20 lb. per acre of Chel-138) for effective control of chlorosis. Bould,<sup>16, 17</sup> working with chlorotic pears, obtained no visual response to soil applications of iron lignin sulphonate when used at rates supplying 20–40 g. of Fe per tree. Furthermore he failed to observe any response to surface and sub-soil applications of two iron tannates, when applied at the same rates as the iron lignin sulphonate.

### Stability of iron chelates

The stability constant of  $\text{Fe}^{3+}$ -EDTA ( $\log K = 25$ ) indicates that it should be extremely stable, yet dilute aqueous solutions slowly decompose in the presence of light; ferric iron is first reduced to ferrous iron and subsequently precipitated as ferric hydroxide. This was first shown by Jones & Long<sup>22</sup> and independently by Hill-Cottingham.<sup>23</sup> In moderately acid solutions Fe-EDTA is very stable, but with increase in pH above 6.0 and in the presence of divalent cations such as calcium its stability rapidly decreases; iron is replaced by calcium and precipitated as ferric hydroxide. On the grounds of stability it is clear, therefore, why Fe-EDTA was effective in controlling copper-induced chlorosis in acid soils ( $\text{Fe}^{3+}$ -EDTA is very much more stable than all other nutrient metal chelates at pH 4.0) and why it was less effective, and sometimes ineffective, on calcareous soils. The variable results obtained with Fe-HEEDTA and Fe-DTPA chelates that are known to be stable in alkaline solutions, were at first very puzzling. However, the soil studies of Stewart & Leonard,<sup>24</sup> Lunt *et al.*<sup>25</sup> and Hill-Cottingham & Lloyd-Jones<sup>26</sup> have helped to clarify some of these anomalous field results. These studies showed that when Fe-EDTA, Fe-HEEDTA and Fe-DTPA are added to calcareous clay soils two main reactions take place. The first, which is rapid, involves sorption of some of the chelate molecules by the clay, thus impeding their movement in the soil. The second, which is slower but nevertheless much faster than the reaction in nutrient solutions of similar pH, entails the loss of iron and its replacement by calcium. Alkaline clays appear to catalyse this second reaction.

These findings emphasize the importance of transporting iron chelates to the roots as quickly as possible after application, thus confirming the field observations of Bould.<sup>16, 17</sup>

The more recent iron chelates, Chel-138 and Fe-CDTA, were shown by Wallace *et al.*<sup>18</sup> to be much more stable in a calcareous soil (32% CaCO<sub>3</sub>) than any of the other iron chelates tested. Chel-138 was not sorbed by clay and both chelates appeared to be resistant to biological decomposition.

#### Absorption and utilization of iron chelates by plants

Iron chelates are effective in correcting iron chlorosis, although the iron is present in a non-ionized form. The question arises, therefore, do they release iron to stronger chelating agents at the root surface by a process of ion exchange, or are they absorbed in the molecular form and translocated as such to the leaves? By using <sup>14</sup>C-tagged Fe-EDTA Stewart & Leonard<sup>24</sup> concluded that the iron is absorbed in the molecular form: the same conclusion was reached by Wallace & North<sup>27</sup> using <sup>15</sup>N-labelled Fe-EDTA. Weinstein *et al.*<sup>28</sup> produced further evidence that EDTA is absorbed, by employing a divided-root technique. Ionic iron, in a complete nutrient solution at pH 7.0, was supplied to one half of the root system. The iron was absorbed but was inactivated after entry into the plant, causing a chlorosis of the leaves. When Na<sub>2</sub>EDTA was supplied through the other half of the root system it chelated the inactivated iron and the chlorosis disappeared.

Wallace *et al.*<sup>29</sup> grew avocado plants in nutrient solutions containing Fe-EDTA and analysed the leaves for both iron and EDTA. They showed that EDTA was present in the leaves and, on a 1 : 1 molar basis, it accounted for at least 70% of the iron present. Furthermore the experiments showed that EDTA persists in the leaves for some considerable time.

Holmes & Brown<sup>19</sup> used <sup>14</sup>C-labelled Fe-DTPA and by means of autoradiographs showed an even distribution of <sup>14</sup>C in the tops which increased linearly with rate of application to the roots. Radio-iron accumulated in the leaf and flower primordia without a corresponding increase of <sup>14</sup>C, which suggests the separation of iron from chelate within the plant.

The evidence, so far, indicates that iron chelates of the polyamino-polycarboxylic type are absorbed in the molecular form. How, therefore, does the plant remove the iron from these stable complexes?

The order in which metallic cations are complexed depends on pH, and on the nature and concentration of precipitating anions. This should be borne in mind when using the stability constants of iron chelates for predicting possible reactions with metals. There is, however, a very large difference between the stability of ferric and ferrous chelates, for example  $\log K$  for Fe<sup>3+</sup>-EDTA = 25.1 and  $\log K$  for Fe<sup>2+</sup>-EDTA = 14.2. Hill-Cottingham<sup>23</sup> has shown that Fe-EDTA, Fe-HEEDTA and Fe-DTPA all undergo photo-reduction and he suggests that this may be the key to the release of iron in the leaves.

#### Foliar sprays

In view of the instability of some iron chelates in calcareous soils and the high cost of soil treatment, a number of workers have tried to apply them as foliar sprays. Bould<sup>16, 17</sup> found that for a number of chelates the upper concentration which could be used without causing leaf damage to pears lay between 0.05 and 0.1%; several consecutive sprays at this concentration were necessary to give a complete control of chlorosis. Of the chelates tested Fe-CDTA and Fe-HEEDTA appeared to be the most effective. In other experiments with fruit, one spray of 0.1% Fe-DTPA gave almost complete control of chlorosis in wall peaches. Plums and redcurrants were less susceptible to spray damage than pears. In applying iron chelate sprays, as with all nutrient sprays, it is essential to use a wetting agent and to wet thoroughly both upper and lower leaf surfaces. Translocation of absorbed iron is limited, hence the need for more spraying as new growth is made. The control of chlorosis, except possibly by Fe-CDTA, is seasonal.

### Growth-promoting effects of chelating agents

Holmes & Brown<sup>19</sup> noticed that Chel-138 had a significant effect on the general micro-element content of soya-bean plants and they suggested that the chelate might have affected the metabolism of the root. Recently, Heath & Clark<sup>30, 31</sup> observed that EDTA at  $10^{-5}M$  stimulated the growth of wheat coleoptile sections. Bennet-Clark<sup>32</sup> also noticed that EDTA at  $10^{-5}$ – $10^{-3}M$  can act as a growth promoter and he suggested that it might be concerned with the calcium metabolism of the cell wall. Weinstein *et al.*<sup>33</sup> also found stimulating effects of EDTA at  $10^{-5}M$  on sunflower seedlings. The response to Fe-EDTA, however, was much reduced, and from their work it would appear that the growth-promoting effects are associated only with the uncomplexed chelating agent. This can be interpreted as supporting the concept that growth is promoted by removal of calcium from the cell wall by the chelating agent. Fawcett *et al.*,<sup>34</sup> using the free acid and sodium salts of EDTA, concluded that the growth-promoting effects shown by these substances, at  $10^{-5}$ – $10^{-3}M$ , may well depend upon changes in membrane permeability and cell-wall structure resulting from sub-acute toxicity, and that they are not acting as true growth promoters.

### Conclusions

Considerable advances have been made in our knowledge of the chemical properties and use of iron chelates in plant nutrition during the past few years, but our understanding of the behaviour of chelates within the plant is very limited. It is clear from soil studies that the iron chelates at present commercially available have serious limitations when used as soil dressings on calcareous soils, although the new chelates, still in the experimental stage, appear more promising. If the latter compounds can be produced at economic costs, it should be possible in the future to control lime-induced chlorosis by soil dressings. Until these new chelates are available care must be taken to use the existing chelates in the appropriate manner, i.e., by soil application to ensure rapid transport to the roots, or by foliar application.

### References

- <sup>1</sup> Stewart, I., & Leonard, C. D., 'Mineral Nutrition of Fruit Crops', 1954, p. 775 (Somerville, N.J.: Somerset Press)
- <sup>2</sup> Gile, P. L., & Carrero, J. O., *J. agric. Res.*, 1916, **7**, 503
- <sup>3</sup> Reed, H. S., & Haas, A. R. C., *Bot. Gaz.*, 1924, **77**, 290
- <sup>4</sup> Heck, W. W., & Bailey, L. F., *Plant Physiol.*, 1950, **25**, 573
- <sup>5</sup> Hutner, S. H., Provasoli, L., Schatz, A., & Haskins, C. P., *Proc. Amer. phil. Soc.*, 1950, **94**, 152
- <sup>6</sup> Jacobson, L., *Plant Physiol.*, 1951, **26**, 411
- <sup>7</sup> Woltz, S. S., quoted by Stewart & Leonard<sup>1</sup>
- <sup>8</sup> de Kock, P. C., *Soil Sci.*, 1955, **79**, 167
- <sup>9</sup> de Kock, P. C., & Strmecki, E. L., *Physiol. Plant.*, 1954, **7**, 503
- <sup>10</sup> Stewart, I., & Leonard, C. D., *Citrus Mag.*, 1952, **14**, 22
- <sup>11</sup> Reuther, W., & Smith, P. F., *Florida St. hort. Soc. Quart.*, 1952, **65**, 62
- <sup>12</sup> Leonard, C. D., & Stewart, I., *Proc. Amer. Soc. hort. Sci.*, 1953, **62**, 103
- <sup>13</sup> Chaberek, S., & Bersworth, F. C., *Science*, 1953, **118**, 280
- <sup>14</sup> Wallace, A., North, C. P., Kofranek, A. M., & Lunt, O. R., *Calif. Agric.*, 1953, **6**, 13
- <sup>15</sup> Leonard, C. D., & Stewart, I., *Florida St. hort. Soc. Quart.*, 1954, **66**, 49
- <sup>16</sup> Bould, C., *Nature, Lond.*, 1955, **175**, 90
- <sup>17</sup> Bould, C., *Annu. Rep. Long Ashton Res. Sta.*, 1955, **87**
- <sup>18</sup> Wallace, A., Mueller, R. T., Lunt, O. R., Ashcroft, R. T., & Shannon, L. M., *Soil Sci.*, 1955, **80**, 101
- <sup>19</sup> Holmes, R. S., & Brown, J. C., *Soil Sci.*, 1955, **80**, 167
- <sup>20</sup> Gasser, R., & Müller, G., 2nd Symposium on Plant Analysis and Fertilizer Problems, Paris, 1956, in press
- <sup>21</sup> Wallace, A., & Ashcroft, R. T., *Soil Sci.*, 1956, **82**, 233
- <sup>22</sup> Jones, S. S., & Long, F. A., *J. phys. Chem.*, 1952, **56**, 25
- <sup>23</sup> Hill-Cottingham, D. G., *Nature, Lond.*, 1955, **175**, 347
- <sup>24</sup> Stewart, I., & Leonard, C. D., Proc. int. Conf. on the peaceful Uses of atomic Energy, 1956, Vol. 12, 159 (New York: United Nations)
- <sup>25</sup> Lunt, O. R., Hemaidan, N., & Wallace, A., *Soil Sci. Soc. Amer. Proc.*, 1956, **20**, 172
- <sup>26</sup> Hill-Cottingham, D. G., & Lloyd-Jones, C. P., *Plant Soil*, in press
- <sup>27</sup> Wallace, A., & North, C. P., *Calif. Agric.*, 1953, **7**, 10
- <sup>28</sup> Weinstein, L. H., Purvis, E. R., Meiss, A. N., & Uhler, R. L., *J. agric. Food Chem.*, 1954, **2**, 421
- <sup>29</sup> Wallace, A., North, C. P., Mueller, R. T., Shannon, L. M., & Hemaidan, N., *Proc. Amer. Soc. hort. Sci.*, 1955, **65**, 9
- <sup>30</sup> Heath, O. V. S., & Clark, J. E., *Nature, Lond.*, 1956, **177**, 1118
- <sup>31</sup> Heath, O. V. S., & Clark, J. E., *Nature, Lond.*, 1956, **178**, 600
- <sup>32</sup> Bennet-Clark, T. A., 'The Chemistry and Mode of Action of Plant Growth Substances', 1956, p. 284 (London: Butterworth Publications)
- <sup>33</sup> Weinstein, L. H., Meiss, A. N., Uhler, R. L., & Purvis, E. R., *Nature, Lond.*, 1956, **178**, 1188
- <sup>34</sup> Fawcett, C. H., Wain, R. L., & Whightman, F., *Nature, Lond.*, 1956, **178**, 972

**Discussion**

*Dr. A. M. Smith* (Edinburgh): Is lignin sulphonate available in this country?

*Dr. Bould*: The product in liquid form containing 5% iron is marketed by Norman Evans & Rais, Ltd.

*Dr. N. H. Pizer* (N.A.A.S., Cambridge): How can deficiencies in strawberries or blackcurrants be treated?

*Dr. Bould*: Whereas trees can be treated by soil injection, strawberries and blackcurrants needed a foliar spray, which gives control for one season only. To prevent injury from spray a wetting agent must be added and the spray should be used immediately after being made up because it is broken down by the action of light. A spray containing 0.1% active material is usually used.

*Mr. F. C. Cooke* (Editor, 'World Crops'): In view of the high cost of the recommended forms of chelated iron, what is the special merit they have over ferrous sulphate in the form of foliar sprays?

*Dr. Bould*: The whole chelate molecule is taken up by the plant and translocated, which gives a more even distribution; ferrous sulphate produces a spotted effect and more than one application is usually required.

*Dr. Pizer*: Is there any derangement of metabolism after uptake of chelating agents by the plant; were the minor constituents affected?

*Dr. Bould*: An excess of the chelating agent may adversely affect the balance of trace elements within the plant.

*Mr. R. C. Voss* (West of Scotland College): Is there a chance that chelates will increase the toxicity of other trace elements?

*Dr. Bould*: This is not the case with copper but much depends on the pH.

*Dr. R. L. Mitchell* (Macaulay Institute): With EDTA the divalent cations, nickel and cobalt, are rejected by the plant roots. Trivalent cations, such as gallium and indium, when chelated, can be taken up in large amounts, yet they do not produce chlorosis.



## ASPECTS OF MOLYBDENUM AS AN ESSENTIAL NUTRIENT FOR HIGHER PLANTS\*

By E. J. HEWITT

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The observation,<sup>1</sup> later shown to be of general importance,<sup>2-4</sup> that nitrate or oxidizing compounds accumulate in plants deprived of molybdenum, led to the conclusive demonstration of the rôle of molybdenum in the nitrate reductase enzyme in plants<sup>5</sup> and micro-organisms.<sup>6, 7</sup>

It is of interest to enquire if this specific function comprises the entire rôle of molybdenum as an essential plant nutrient. No conclusive answer is possible at present but circumstantial evidence is reviewed here and some other relevant aspects of molybdenum metabolism are discussed.

The criteria proposed by Arnon<sup>8</sup> by which the essentiality of an element may be judged are briefly: (a) omission of the element must result in abnormal development, failure to reproduce or cessation of growth, (b) the element cannot be substituted by one or more other elements, (c) the essential action must be direct, i.e., it is not the result of an antagonism in the uptake or distribution of another element exerting toxic effects. If omission of the element whose essential nature is being investigated results in the suspension or diversion of specific metabolic activity *without marked detriment to the growth of the whole organism* the element does not qualify as essential.

### Growth of molybdenum-deficient plants with nitrate

The criteria are clearly and absolutely satisfied when the effects of molybdenum deficiency are considered for plants grown entirely with nitrate in regard both to the specific rôle in nitrate reduction and the effects on growth responses outlined below.

All molybdenum-deficient plants grown with nitrate have shown very similar symptoms.<sup>9</sup> These include chlorosis (yellow-green, yellow or orange mottling of leaves) followed by marginal curling, wilting, necrosis and leaf withering, often immediately preceded by water-soaking of some areas by escaped cell fluids. Cotyledons may remain green and turgid for several weeks under these conditions. Symptoms usually appear first in old leaves but not always in the oldest or first true leaf. They progress regularly to younger leaves until the growing point is killed and the plant dies. Flowers wither or are suppressed. Rapid and total recovery is possible on adding molybdenum at any time.

These symptoms are associated with low yield, often less than 5% of normal plants, and with the accumulation of nitrate<sup>10, 11</sup> which may attain more than 15% of total dry material. The contents of protein, total soluble organic nitrogen and chlorophyll are abnormally low. The mottled areas that are low in chlorophyll are correspondingly lower in molybdenum and higher in nitrate than adjacent greener areas.<sup>9, 11</sup>

In one group of plants, the brassicas, other symptoms associated with molybdenum deficiency may also occur. These symptoms are known as 'whiptail' in cauliflower and broccoli<sup>11, 12</sup> and appear in analogous form in other species.<sup>9, 13</sup> Their occurrence is related to molybdenum supply in a characteristic manner which is important in assessing the question of molybdenum requirement.

In cauliflower the symptoms start always as one or more small rounded translucent areas between major veins near the midrib of one or two young leaves (usually 6-15 cm. long): these areas become yellow or totally chlorotic, necrotic and rapidly perforate. As the leaf continues to expand they leave enlarged irregular holes. In successive younger leaves the chlorotic areas appear less regular in shape and occur nearer to, or in, the marginal regions. The lamina here is unable to expand and as the leaves elongate the margins become wavy, irregular and defective. Leaves with little or no lamina are finally produced but they may

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still elongate considerably producing the 'whiptail' effect. The growing points of the youngest leaves and of the stem apex are finally killed. They lose their cell contents and dry up, leaving smooth, rudimentary stumps. Old leaves, by contrast, remain attached to the plant and appear turgid, green and normal for several weeks, except for a tendency to crack easily. Plants are unable to make normal growth. In acutely deficient plants flowering is suppressed and therefore reproduction is totally prevented.

The occurrence of these symptoms in direct response to omission of molybdenum would therefore comprise strong evidence of essentiality in terms of the criteria already specified.

#### Effect of molybdenum deficiency with nitrogen sources other than nitrate

In order to establish the requirement for molybdenum by plants grown in the absence of nitrate or with alternative sources of nitrogen, evidence of abnormal development, marked by depressed yield, reproductive failure or harmful metabolic irregularity must be produced. Experiments have, therefore, been made with cauliflower and tomato grown in sand or water culture, using several different nitrogen sources with or without added molybdenum, and taking the usual precautions to minimize extraneous sources of contamination.<sup>14, 15</sup> The results have been compared with those observed from the use of nitrate and the following points have been established:

(1) The symptoms of mottling, wilting, scorching and progressive withering of old leaves are associated with the provision of nitrogen as nitrate. Inclusion of substantial amounts of nitrogen as ammonium nitrogen or nitrite in addition to nitrate does not eliminate these symptoms although they may be modified owing to less marked loss of chlorophyll.<sup>15, 16</sup>

(2) Molybdenum-deficient plants grown with ammonium sulphate, ammonium citrate, ammonium nitrite, glutamic acid or urea contain at least normal and possibly higher levels of chlorophyll than comparable plants given molybdenum. With nitrite, slight but not significant decreases have sometimes been recorded.<sup>15, 16</sup>

It is evident that mottling, withering and loss of chlorophyll in molybdenum-deficient plants are associated with the provision and probably the marked accumulation of unassimilated nitrate and do not occur when other nitrogen sources *only* are given.

Molybdenum has no direct rôle, therefore, in chlorophyll synthesis.

(3) Nitrate accumulation appears to be associated also with abnormally high respiratory quotients in molybdenum-deficient cauliflower.<sup>17</sup> Plants grown with other sources of nitrogen appeared to have a normal respiration.

(4) Symptoms of 'whiptail' in cauliflower as described above have been regularly observed in molybdenum-deficient plants given all sources of nitrogen mentioned,<sup>15</sup> and in the absence of nitrate these are the only symptoms to occur; the characteristic chlorotic mottling, wilting and necrosis of old leaves are absent. Molybdenum levels in sand culture required to produce the 'whiptail' symptoms are estimated to be between 0.000005 and 0.00005 p.p.m.

The occurrence of 'whiptail' in plants grown with nitrate was unpredictable in earlier experiments and often appeared to be associated with partial recovery from initially severe symptoms of mottling and leaf collapse.<sup>1, 13</sup> It is now recognized<sup>18</sup> that the production of 'whiptail' in plants grown with nitrate depends upon the supply of a strictly limited amount of molybdenum corresponding to about 0.00002–0.0001 p.p.m. in sand cultures, or to the uptake during the early part of the experiment of just sufficient molybdenum provided by accidental slight contamination to permit assimilation of a limited amount of nitrate, temporary recovery and renewal of growth.

Plants grown all the time with nitrate at the lowest levels of 0.00005 p.p.m. of Mo do not show 'whiptail' symptoms and remain at the mottling and leaf necrosis stage, whilst those grown with nitrate at 0.00005 p.p.m. of Mo develop 'whiptail', show little or no mottling and often do not contain appreciable amounts of free nitrate.<sup>10, 12</sup>

(5) Molybdenum-deficient cauliflower grown with ammonium compounds, nitrite, urea or glutamic acid contain usually as much amino-nitrogen, protein and free adenine as those grown with these sources or with nitrate in the presence of molybdenum.<sup>19, 20</sup> It appears also that molybdenum-deficient plants grown with ammonium compounds, urea or glutamic acid and

often also those grown with nitrite contain abnormally high concentrations of several amino-acids, of which arginine is the most notable and consistently found. Effects with nitrate are not consistent possibly because the production of all amino-acid compounds is limited by molybdenum deficiency and this will tend to mask any trends of an opposite nature which can be detected when other nitrogen sources are given.

'Whiptail' symptoms, therefore, appear to occur independently of the effect of molybdenum on nitrate assimilation. Indeed, when nitrate alone is given it seems that their occurrence is conditional upon a minimum level of nitrate assimilation, permitted by a low but not negligible molybdenum supply. It is concluded that the incidence of 'whiptail' in molybdenum-deficient plants is associated with the presence of sufficient organic nitrogen, and is not a direct result of injury by nitrate accumulation. The possibility that 'whiptail' symptoms are caused by the production in toxic amounts of some specific organic nitrogen compound in certain tissues at particular stages of development of molybdenum-deficient plants cannot be excluded at present.

(6) Yields of molybdenum-deficient cauliflower are markedly decreased<sup>15</sup> when they are grown with ammonium compounds, urea, glutamic acid or nitrite.

Although such decreases might occur indirectly, owing to the loss of effective lamina tissue in plants affected by 'whiptail', parallel experiments with tomato<sup>16</sup> have shown that yields are similarly significantly decreased in this plant although no leaf malformation was observed.

Tomato plants have rarely shown any symptoms of molybdenum deficiency in the absence of nitrate, although occasional mottling and leaf wilting, distinct from that associated with the use of nitrate, have been observed.<sup>16, 21</sup> The decreases in growth appear, therefore, to result from some defect in metabolism which does not directly or readily lead to symptoms of injury in this plant. It is also clear<sup>16</sup> that the quantitative molybdenum requirements of tomato are significantly lower with ammonia compounds, urea, glutamic acid or nitrite than with nitrate. In this connexion Ishioka & Arnon<sup>22</sup> have concluded that in *Scenedesmus obliquus* there is in fact *no* molybdenum requirement in the presence of ammonium compounds or urea, in contrast to that demonstrated with nitrate.

This conclusion is, however, not capable of proof, and in terms of the interpretation of negative results set out earlier by Arnon<sup>8</sup> it cannot be accepted as beyond possible doubt. It may be significant, however, that Arnon & Wessel<sup>23</sup> found that the same organism requires vanadium in addition to, or in the presence of, molybdenum. This requirement is apparently absolute but has not so far been reported for other plants. It is conceivable that in *Scenedesmus* vanadium has replaced molybdenum in some other function that it appears to fulfil in other micro-organisms and in higher plants. It is relevant also to note that vanadium is able to replace, in varying degrees ranging from complete to nil, molybdenum in nitrogen fixation by different strains of *Clostridium butyricum*.<sup>24</sup>

#### Effects of molybdenum on ascorbic acid and sugars

Molybdenum deficiency causes marked decreases in ascorbic acid concentrations in all plants examined.<sup>25</sup> Injection of molybdenum into deficient plants leads to increased concentrations within 24 hours and normal levels are restored in 3-5 days.<sup>15, 25</sup>

The effect of molybdenum on ascorbic acid content is proportional to molybdenum supply<sup>26</sup> and independent of the nitrogen supply in tomato and cauliflower.<sup>15, 16</sup> There does not appear to be a consistent or marked effect on the ratio of ascorbic to dehydroascorbic acids in these plants.<sup>15, 16, 26</sup>

Contents of total and reducing sugars also appear to be decreased by molybdenum deficiency in cauliflower.<sup>10</sup> These changes are mainly independent of nitrogen supply<sup>15</sup> but are apparently complex, since in the presence of nitrate extremely low molybdenum levels may result in higher sugar contents than those found in plants with 'whiptail'.<sup>10</sup> The changes in sugar content do not appear to be the direct cause of low ascorbic acid content in molybdenum-deficient plants, and both are independent of chlorophyll content.

The facts presented here show that omission of molybdenum causes a disturbance of metabolism under conditions where reduction and assimilation of nitrate are not directly limiting factors either in growth or in gross protein synthesis, and where possible injury from nitrate accumulation does not appear significant or likely. This disturbance is associated with limited

growth, reproductive failure, low ascorbic acid concentrations and, in one group of plants, a specific type of tissue breakdown. It is therefore concluded in terms of the criteria mentioned above that molybdenum is required by higher plants, and, by inference from work with *Aspergillus* and *Neurospora*,<sup>27</sup> by micro-organisms also, regardless of the presence or absence of nitrate as a source of nitrogen. It is therefore necessary to consider possibilities that may account for this general requirement for molybdenum.

#### Other enzyme systems requiring molybdenum

There is at present no evidence of enzyme systems other than nitrate reductase in higher plants for which molybdenum is specifically needed, though these may be discovered in time. The hydrogenase complex in *Clostridium pasteurianum*<sup>28</sup> appears to require molybdenum in certain circumstances, but it is unlikely that such an enzyme system occurs in higher plants.

Xanthine oxidases<sup>29, 30</sup> which also contain or require molybdenum when functioning as systems transferring electrons singly to nitrate or cytochrome *c*, have not been found so far in any plant tissues. Aldehyde oxidase enzymes of animal tissues<sup>31</sup> are similar to xanthine oxidase in some respects and also involve molybdenum. An aldehyde oxidase system occurs in potato tubers but is not widely distributed in other plants so far examined. The potato enzyme also may be a metallo-protein and can transfer electrons from aldehydes to nitrate. Investigations in progress at Long Ashton<sup>32</sup> show that its activity may be increased by molybdate and also by flavin nucleotides, but these effects cannot always be reproduced. However, the apparently restricted distribution of the enzyme, which has not been detected in cauliflower or tomato, excludes it at present as an explanation for the general molybdenum requirement that may be inferred.

#### Relation of molybdenum to phosphorus and silicon metabolism

Molybdate catalyses the hydrolysis of several phosphate and pyrophosphate esters.<sup>33</sup> It is unlikely, however, in view of the wide distribution of specific and non-specific phosphatases that this property would have biological significance or advantage at normal physiological concentrations of molybdenum.

Molybdenum-deficient cauliflower and tomato plants have a higher ratio of inorganic to organic or total phosphate than normal plants<sup>34</sup> and this highly significant effect appears to be independent of nitrogen source as indicated in Table I. Addition of molybdate to deficient tomatoes results in the rapid conversion of inorganic into organic phosphate compounds<sup>35</sup> at

Table I

Effect of molybdenum and nitrogen treatments on ratio inorganic/total phosphorus in leaves of cauliflower  
Mean values for 6 estimations between 25/8/52 and 17/9/52<sup>15</sup>

Nitrogen supply and nutrient supplements	Deficiencies of Mo (0.000005–0.00005 p.p.m.)	Mo added (total) (0.05 p.p.m.)
Nitrate	0.853	0.761
Nitrate and citrate	0.559	0.587
Nitrate and sucrose	0.616	0.445
Nitrate and calcium carbonate	0.627	0.425
Nitrate and arginine	0.558	0.489
Nitrate and nitrite	0.620	0.558
Nitrite	0.714	0.532
Nitrite and citrate	0.664	0.607
Nitrite and malate	0.765	0.569
Ammonium nitrite	0.538	0.549
Ammonium nitrate	0.808	0.671
Ammonium sulphate and calcium carbonate	0.489	0.453
Ammonium citrate	0.638	0.702
Ammonium malate	0.695	0.524
Urea	0.666	0.503
Urea and citrate	0.634	0.633
Urea and malate	0.661	0.505
Glumatic acid	0.665	0.444
Mean	0.650	0.553

Results with deficiency of Mo > addition of Mo,  $p < 0.001$

present unidentified. An attempt was made to separate some of the phosphorus fractions present in trichloroacetic acid extracts of leaves from cauliflower plants grown with different nitrogen and molybdenum supplies. The barium fractionation method of Lepage<sup>36</sup> was used. [The trichloroacetic acid extracts are treated with barium acetate and alcohol at pH 8.4. The precipitate is separated from the alcohol-soluble fraction and refractionated to give an *o*-IN-HCl-insoluble fraction, a water-insoluble fraction and an alcohol-insoluble fraction. Total inorganic and acid-labile phosphorus (7-min. hydrolysis in *N*-HCl) are determined in each.] The method was concluded to be unsatisfactory with plant materials owing to interference by adsorbed pigments and high concentrations of inorganic phosphate, and to the production of an acid-insoluble barium fraction with which is probably associated the greater part of the adenosine pyrophosphate content of some plant tissues.<sup>37</sup> Results in Table II below show that, in spite of these limitations, the proportion of inorganic P in the total P was, with one exception, increased by molybdenum deficiency and the total organic phosphorus was correspondingly decreased. The proportion of organic phosphorus in the trichloroacetic-acid-soluble fraction was consistently decreased by molybdenum deficiency. The proportion of organic phosphorus in separate water-soluble, alcohol-soluble and alcohol-insoluble fractions, with two exceptions, and also the proportion of acid-labile phosphorus with two exceptions, were also decreased by molybdenum deficiency.

The lower content of organic or esterified phosphate in molybdenum-deficient plants could be explained in two ways, (1) that molybdenum is required in some aspect of phosphorylation, or (2) that it acts as a phosphatase inhibitor at normal physiological concentrations.

Table II

*Effect of molybdenum supply on phosphorus fractions in cauliflower, given with different nitrogen treatments;  $\mu$ g. of P per g. fresh wt.*

Treatments	Total P (ash analysis)	Acid- in- soluble P	Trichloroacetic-acid-soluble fractions						Organic P	Total acid- soluble P
			Barium fractions (organic P)				In- organic P	7-min. acid- labile P		
			Alcohol- sol.	Alcohol- insol.	Water- insol.	Acid- insol.				
Nitrate										
+ Mo	889	344	68	52	35	49	357	34	204	576
- Mo	767	343	66	34	22	18	275	24	140	428
Nitrite										
+ Mo	1132	315	69	41	110	24	577	173	244	823
- Mo	746	173	42	25	39	24	449	39	130	579
Nitrate and nitrite										
+ Mo	714	422	70	35	37	32	173	49	174	328
- Mo	820	343	92	17	18	48	316	30	175	605
Ammonium sulphate										
+ Mo	893	342	120	20	25	53	318	22	218	536
- Mo	860	288	92	15	20	45	390	39	172	560
Urea										
+ Mo	658	231	63	17	15	44	280	28	139	419
- Mo	995	283	114	19	52	41	495	18	226	712
Ammonium citrate										
+ Mo	890	207	71	12	48	60	452	72	191	641
- Mo	1128	320	63	14	36	73	623	109	186	806
Ammonium malate										
+ Mo	7034	242	88	11	58	31	260	107	188	421
- Mo	1196	465	127	12	50	43	612	125	232	868

+ = addition of Mo

- = deficiency of Mo

An inhibitory effect of molybdate on acid phosphatase activity has been demonstrated for potato<sup>38</sup> and for tomato.<sup>39</sup> In tomato, concentrations of the order of those present naturally exerted a significantly inhibitory effect. On the other hand, attempts to show any effect of molybdenum status of, or infiltration into, cauliflower leaves were unsuccessful.<sup>40, 41</sup>

Xanthine and aldehyde oxidases require phosphate or silicate in addition to molybdenum for activity in single electron reactions,<sup>29-31</sup> and nitrate reductase of *Neurospora* is now found to be similar.<sup>42</sup> Further, aldehyde oxidase of liver can reduce free phosphomolybdic or silicomolybdic acid to yield molybdenum blue.<sup>43</sup> This is thought to explain the inability of fresh sodium molybdate solutions alone to activate the xanthine and aldehyde oxidase enzymes. The most likely function of phosphate or of any radical able to form a complex with molybdenum is to adjust the oxidation-reduction potential of the molybdenum couple to a value that permits electron-transfer from the flavin or other active group in the enzyme. This reaction provides the chemical basis for a possible phosphorylation mechanism in which phosphate transfer from a phosphomolybdic complex could be coupled to valency change during reduction of hexavalent molybdenum, in a molybdenum-containing flavoprotein. This would comprise oxidative phosphorylation at the flavin level, whilst the hexavalent molybdenum required for primary phosphate combination would be regenerated by oxidation by the single electron acceptor. Mahler<sup>44</sup> has discussed the possibility also that metallo-flavoproteins may be widely involved in coupled phosphorylation reactions, but no evidence is available at present. Attempts to reduce free phosphomolybdate or silicomolybdate complexes with potato aldehyde oxidase or cauliflower extracts containing highly active nitrate reductase have so far been unsuccessful.

Albaum, Ogur & Hirschfeld<sup>37</sup> found that adenosine pyrophosphates closely related to ATP (adenosine triphosphate) were concentrated in the acid-insoluble barium fraction from mung bean extracts. Using a method based on that described,<sup>37</sup> it was found that compounds able to transfer phosphate to glucose in the presence of hexokinase, though at a slower rate than from ATP, were present in the acid-insoluble barium fractions obtained from the cauliflower leaves. Free 5'-adenylic acid and total pyrophosphate-combined derivatives were found to occur also in the other barium fractions described above. These compounds were identified and estimated spectrophotometrically<sup>45, 46</sup> in each fraction by observation of the changes in optical density at 265 and 240 m $\mu$  and confirmation obtained of the isobestic point at 248 m $\mu$  on treatment with specific muscle 5'-adenylic acid deaminase<sup>46</sup> either alone or in conjunction with potato adenosine pyrophosphatase,<sup>46</sup> which was sufficiently free from phosphatase for analytical use. Tests with chromatographically pure 5'-adenylic acid and an ATP-ADP (adenosine diphosphate) mixture yielded 95% recoveries during the reaction period of about 20 minutes. There was no appreciable inhibition in the presence of the plant materials.

The results on cauliflower leaf extracts are given in Table III as total adenylic acid combined in the pyrophosphate form and as free adenylic acid. There was a marked tendency for these compounds to be present in higher concentrations in extracts from molybdenum-deficient plants. There was, however, no clear evidence that the proportions of pyrophosphate to free adenylic acid were affected by molybdenum supply. The increased concentrations of adenosine phosphates might also occur if nuclease enzymes were more active in tissues or during preparation of the extracts under conditions of molybdenum deficiency.<sup>46a</sup>

#### Effect of molybdenum supply on enzyme complement

Nason and collaborators<sup>47-49</sup> have shown that mineral deficiencies may cause large changes in the activity of several enzymes that are not activated by normal concentrations of the deficient metal. These changes often appear as great *increases* in the activities of various enzymes, e.g., of diphosphopyridine nucleotidase (DPN-ase) in absence of zinc, or of isocitric dehydrogenase of tomato in the absence of copper.

It is possible, therefore, that molybdenum deficiency also causes analogous changes in one or more enzymes. Phosphatase activity has already been mentioned in a different respect. Polyphenol oxidase and peroxidase activities per mg. of protein may be doubled in molybdenum-deficient tomatoes<sup>48</sup> grown with nitrate as a source of nitrogen. In cauliflower, an ascorbic acid oxidase associated with the cell wall,<sup>50</sup> though not consistently affected by molybdenum

Table III

Effect of molybdenum supply on adenosine 5'-pyrophosphates and free 5'-adenylic acid in barium fractions obtained from trichloroacetic acid extracts of cauliflower leaves ( $\mu\text{g. per g. of fresh weight as 5'-adenylic acid}$ )

Treatments		Barium fractions							
Nitrogen supply	Mo	Acid-insoluble		Water-insoluble		Alcohol-insoluble		Total	
		Adenosine pyro-phosphate	Adenylic acid	Adenosine pyro-phosphate	Adenylic acid	Adenosine pyro-phosphate	Adenylic acid	Adenosine pyro-phosphate	Adenylic acid
Nitrate	+	3.80	0	0.79	0	0	0	4.59	0
	-	5.30	0	2.78	1.39	9.65	0	17.73	1.39
Nitrite	+	3.06	0	0.76	3.70	0	1.05	3.82	4.75
	-	5.80	0	6.60	3.88	5.55	1.42	17.95	5.30
Nitrate and nitrite	+	1.15	0	0.47	0	5.74	0	7.36	0
	-	6.65	1.18	3.02	0	0	2.13	9.67	3.31
Ammonium sulphate	+	14.40	0.96	3.29	2.67	1.53	12.10	19.22	15.73
	-	14.30	0	5.17	2.58	8.10	21.40	27.57	23.98
Ammonium citrate	+	7.90	1.10	2.03	0	1.55	0	11.48	1.10
	-	8.48	1.06	6.71	0.67	0.81	2.82	16.00	4.55
Ammonium malate	+	4.78	0.53	4.10	0	3.16	1.53	12.04	2.06
	-	9.80	2.15	14.10	2.83	0	8.01	23.90	12.99

+ = addition of Mo

- = deficiency of Mo

supply, is often significantly increased in activity per mg. of protein by molybdenum deficiency regardless of nitrogen supply.

Changes such as these might cause metabolic disturbances, e.g., in ascorbic acid metabolism, independent of the type of nitrogen supply and might therefore account in part for a general molybdenum requirement. It is still necessary, however, to explain the specific way in which abnormal enzyme activity of the type described might result from omission of molybdenum.

Some enzymes are adaptive in nature. An adaptive effect of zinc deficiency, for example, on increased production of DPN-ase,<sup>47</sup> can now be understood since alcohol dehydrogenase is greatly decreased at the same time, and this latter enzyme<sup>51</sup> and possibly many other DPN-linked dehydrogenases<sup>52</sup> may be zinc proteins. Failure to produce these enzymes owing to zinc deficiency should increase the concentration of free (unbound) DPN in the cells and might stimulate an adaptive increase in production of DPN-ase.

For molybdenum the only known enzyme in plants is nitrate reductase. Recent work at Long Ashton<sup>32</sup> suggests that nitrate reductase is adaptive in higher plants, as represented by cauliflower. Adaptive production of the enzyme in cauliflower occurs in about two hours in tissues from plants grown with nitrate, nitrite or ammonium sulphate, on addition of molybdenum.<sup>32</sup> Adaptive production also responds to nitrate or nitrite. In molybdenum-deficient tissues grown with ammonium sulphate only, however, the effects of molybdenum alone can still be observed.

The question, therefore, to consider here is whether failure to produce nitrate reductase, which is shown by its non-appearance in cell-free extracts of deficient tissues to which molybdenum is added *subsequently*, may account for a general molybdenum requirement in plants even when nitrate is not utilized for growth. The actual concentration of the enzyme in the cell would not appear, however, to be the determining factor in a mechanism such as that under discussion, since there is no evidence that quite low levels of activity observed in normal plants<sup>41</sup> or fungi<sup>27</sup> grown with ammonium sulphate are associated with any effects suggestive of molybdenum deficiency. On the other hand an inductive *event* that is associated with the capacity to produce the enzyme may be concerned, rather than an adaptive one to regulate its ultimate level from an established production mechanism.

Two independent stimuli, namely molybdenum and nitrate, are shown to be involved in the adaptive appearance of nitrate reductase and it is possible that two different phases of its adaptive production are, in fact, involved. As molybdenum is a constituent<sup>6</sup> and nitrate is

only the substrate of the enzyme, the metal may have a strictly inductive effect, whilst presence of the substrate may determine only the extent of final synthesis.

If occurrence or failure of the inductive event rather than the final cell concentration of the enzyme is the factor that interacts with preferential synthesis of other enzymes in the cell, then the general requirement for molybdenum regardless of nitrogen source might be understood. The quantitatively greater requirement in the presence of nitrate might reflect only the facts that with nitrate alone *all* growth is limited by the level of the enzyme, whereas with nitrate together with some other nitrogen source, unassimilated nitrate may be directly injurious.

### Conclusion

In conclusion it appears that molybdenum is required by higher plants and fungi regardless of the supply of nitrate nitrogen, but that in the only instance so far for which a separate or independent vanadium requirement has been observed there is as yet no evidence of a molybdenum requirement in the absence of nitrate. Evidence regarding the probable nature of the general requirement is still lacking, but changes in enzyme systems concerned in phosphate metabolism flavin content, or oxidation-reduction systems likely to affect ascorbic acid, merit further investigation.

### References

- <sup>1</sup> Hewitt, E. J., & Jones, E. W., *J. hort. Sci.*, 1947, **23**, 254
- <sup>2</sup> Mulder, E. G., *Plant & Soil*, 1948, **1**, 94
- <sup>3</sup> Wilson, R. D., & Wareing, E. J., *J. Aust. Inst. agric. Sci.*, 1948, **14**, 141
- <sup>4</sup> Hewitt, E. J., & Bolle-Jones, E. W., *J. hort. Sci.*, 1952, **27**, 257
- <sup>5</sup> Nicholas, D. J. D., & Nason, A., *Plant Physiol.*, 1955, **30**, 135
- <sup>6</sup> Nicholas, D. J. D., & Nason, A., *J. biol. Chem.*, 1954, **207**, 353
- <sup>7</sup> Nicholas, D. J. D., & Nason, A., *J. biol. Chem.*, 1954, **211**, 183
- <sup>8</sup> Arnon, D. I., 'Trace elements in plant physiology', *Lotsya*, 1950, **3**, 31
- <sup>9</sup> Hewitt, E. J., *Soil Sci.*, 1956, **81**, 3
- <sup>10</sup> Agarwala, S. C., & Hewitt, E. J., *J. hort. Sci.*, 1955, **30**, 151
- <sup>11</sup> Stout, P. R., & Meagher, W. R., *Science*, 1948, **108**, 471
- <sup>12</sup> Agarwala, S. C., & Hewitt, E. J., *J. hort. Sci.*, 1954, **29**, 278
- <sup>13</sup> Hewitt, E. J., & Bolle-Jones, E. W., *J. hort. Sci.*, 1952, **27**, 245
- <sup>14</sup> Hewitt, E. J., 'Sand and Water Culture Methods Used in the Study of Plant Nutrition', *Commonw. Bur. Hort., E. Malling*, Tech. Commun. 22, 1952
- <sup>15</sup> Agarwala, S. C., & Hewitt, E. J., *J. hort. Sci.*, 1955, **30**, 163
- <sup>16</sup> Hewitt, E. J., & McCreedy, C. C., *J. hort. Sci.*, 1956, **31**, 284; *Nature, Lond.*, 1954, **174**, 186
- <sup>17</sup> Ducet, G., & Hewitt, E. J., *Nature, Lond.*, 1954, **173**, 1141
- <sup>18</sup> Hewitt, E. J., & Agarwala, S. C., *Nature, Lond.*, 1951, **167**, 733
- <sup>19</sup> Agarwala, S. C., & Williams, A. H., *Annu. Rep. Long Ashton Res. Sta.*, 1951, 66
- <sup>20</sup> Hewitt, E. J., Agarwala, S. C., & Williams, A. H., *J. hort. Sci.*, 1957, **32**, 34
- <sup>21</sup> Hewitt, E. J., & Abbot, A. J., unpublished observations
- <sup>22</sup> Ishioka, P. S., & Arnon, D. I., *Physiol. Plant.*, 1955, **8**, 552
- <sup>23</sup> Arnon, D. I., & Wessel, G., *Nature, Lond.*, 1953, **172**, 1039
- <sup>24</sup> Jensen, H. L., & Spencer, D., *Proc. Linn. Soc. N.S.W.*, 1947, **72**, 73
- <sup>25</sup> Hewitt, E. J., Agarwala, S. C., & Jones, E. W., *Nature, Lond.*, 1950, **166**, 119
- <sup>26</sup> Agarwala, S. C., & Hewitt, E. J., *J. hort. Sci.*, 1954, **29**, 291
- <sup>27</sup> Nicholas, D. J. D., Nason, A., & McElroy, W. D., *J. biol. Chem.*, 1954, **207**, 341
- <sup>28</sup> Shug, A. L., Wilson, P., Green, D. E., & Mahler, H. R., *J. Amer. chem. Soc.*, 1954, **76**, 3355
- <sup>29</sup> Mackler, B., Mahler, H. R., & Green, D. E., *J. biol. Chem.*, 1954, **210**, 149
- <sup>30</sup> Kielley, R. K., *J. biol. Chem.*, 1955, **216**, 405
- <sup>31</sup> Mahler, H. R., Mackler, B., Green, D. E., & Bock, R. M., *J. biol. Chem.*, 1954, **210**, 465
- <sup>32</sup> Hewitt, E. J., unpublished observations
- <sup>33</sup> Weil-Malherbe, H., & Green, R. H., *Biochem. J.*, 1951, **49**, 286
- <sup>34</sup> Hewitt, E. J., *Proc. 13th Int. Hort. Congr., Lond.*, 1952, 1953, **1**, 375
- <sup>35</sup> Possingham, J. V., *Aust. J. biol. Sci.*, 1954, **7**, 221
- <sup>36</sup> Lepage, C. L., in 'Manometric Techniques and Tissue Metabolism', eds. Umbrecht, W. W., Burris, R. H., & Stauffer, J. F., 1949, Chap. XV, p. 185 (Minneapolis: Burgess Co.)
- <sup>37</sup> Albaum, H. G., Ogur, M., & Hirschfield, A., *Arch. Biochem.*, 1956, **27**, 130
- <sup>38</sup> Bailey, J. M., Thomas, C. J., & Whelan, W. J., *Biochem. J.*, 1951, **49**, Proc. vi
- <sup>39</sup> Spencer, D., *Aust. J. biol. Sci.*, 1954, **7**, 151
- <sup>40</sup> Mulder, E. G., *Proc. 6th Int. Microbiol. Congr., Rome*, 1953, **6** (XVIII), 293
- <sup>41</sup> Candela, M. I., Fisher, E. G., & Hewitt, E. J., *Plant Physiol.*, 1957, in press
- <sup>42</sup> Nicholas, D. J. D., & Scawin, J. H., *Nature, Lond.*, 1956, **178**, 1474
- <sup>43</sup> Glenn, J. L., & Crane, F. L., *Biochim. biophys. Acta*, 1956, **22**, 111
- <sup>44</sup> Mahler, H. R., *Proc. 3rd Int. Biochem. Congr., Brussels*, 1955, 252
- <sup>45</sup> Kalckar, H. M., *J. biol. Chem.*, 1947, **167**, 445
- <sup>46</sup> Kalckar, H. M., *J. biol. Chem.*, 1947, **167**, 461
- <sup>46a</sup> Markham, R., private communication
- <sup>47</sup> Nason, A., Kaplan, N. O., & Oldewurtel, H. A., *J. biol. Chem.*, 1953, **201**, 435
- <sup>48</sup> Nason, A., Oldewurtel, H. A., & Propst, L. M., *Arch. Biochem. Biophys.*, 1952, **38**, 1
- <sup>49</sup> Nason, A., *J. biol. Chem.*, 1952, **198**, 643
- <sup>50</sup> Hewitt, E. J., *Plant Physiol.*, 1957, in press
- <sup>51</sup> Vallee, B. L., & Hoch, F. L., *J. Amer. chem. Soc.*, 1955, **77**, 821
- <sup>52</sup> Vallee, B. L., Hoch, F. L., Adelstein, S. J., & Waiker, W. E. C., *J. Amer. chem. Soc.*, 1956, **78**, 5879



**Discussion**

*Dr. Ducet* (Versailles) : What are the anatomical and chemical effects of molybdenum deficiency in whiptail ?

*Dr. Hewitt* : Suspected abnormalities in the cell wall are at present being investigated. At first, I thought that in whiptail, ascorbic acid might be oxidized more readily by cell wall enzymes than in normal tissues ; I have, however, not yet confirmed this.

*Dr. Ducet* asked about the oxidation-reduction potentials in the sap of plants with and without molybdenum.

*Dr. Hewitt* : No data are yet available although it appears that ascorbic acid is more readily oxidized by cell sap from frozen tissues of molybdenum-deficient plants than from normal plants.

*Dr. H. L. Richardson* (Imperial Chemical Industries, Ltd.) : Has *Dr. Hewitt* done any work on the function of molybdenum in clovers ? I realize that the system is a more complex one than that in cauliflower, but the clover problem is very important overseas. In New Zealand, there are molybdenum-deficient soils whose reclamation depends on the use of molybdenum and clovers, along with superphosphate.

*Dr. Hewitt* : *Dr. Nicholas* discusses this in dealing with nutrition of micro-organisms, but it is of interest to note that clovers grown with nitrate still require molybdenum.

*Dr. Williamson* (Aberdeen) : Is the cupping of the leaves, formerly attributed to calcium deficiency, due to direct molybdenum deficiency ?

*Dr. Hewitt* : The cupping is associated with acidity and appears to be mainly dependent on excess manganese. There is an interaction between manganese and molybdenum in acid conditions ; lack of calcium is but one aspect of the soil acidity complex. Leaf-cupping can, however, occur as a result of molybdenum deficiency.

*Dr. Plant* : The balance between molybdenum deficiency and manganese toxicity changes from year to year in acid soils. In field conditions, as the pH falls the symptom of manganese toxicity tends to mask that of molybdenum deficiency.

## RÔLE OF TRACE METALS IN THE NITROGEN METABOLISM OF PLANTS WITH SPECIAL REFERENCE TO MICRO-ORGANISMS\*

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In recent years trace metals have been found to be associated with a number of flavo-proteins, some of which are key enzymes in the nitrogen metabolism of plants. Thus molybdenum is a constituent of nitrate reductase which catalyses the reduction of nitrate to nitrite in bacteria, fungi and green plants. The metal was shown to undergo a valence change involving  $\text{Mo}^V$  and  $\text{Mo}^{VI}$  during the enzymic reduction of nitrate. Nitrite, hydroxylamine and hyponitrite reductases which catalyse the reduction of these compounds to ammonia contain flavin; the first two appear to be stimulated by copper and iron and the last by manganese. The recent identification of a hyponitrite reductase in *Neurospora* strengthens the view that the aliphatic reductive pathway is important in plants. Another flavoprotein enzyme, which reduces dinitrobenzene to nitroaniline in *Neurospora crassa*, is inhibited by metal-chelating agents but the metal concerned has not been identified. Whether the primary reductive sequence from nitrite to ammonia follows an aromatic or an aliphatic route is not known; it may operate in both ways.

Molybdenum, along with other metals including iron, vanadium and tungsten, is important in nitrogen fixation in free living micro-organisms and in root nodule bacteria. It has been suggested that the enzyme hydrogenase which oxidizes molecular hydrogen is important in nitrogen fixation but the fact that it is present in bacteria that require combined N for growth and can be active even when the fixation of nitrogen is low throws doubt on this theory.

Molybdenum has been shown to be a constituent of hydrogenase prepared from *Clostridium pasteurianum*. It is pointed out, however, that iron is required in greater amounts than Mo for nitrogen fixation and hydrogenase activity in *Azotobacter vinelandii* (O strain) and in *Clostridium pasteurianum*. The metals are probably involved in electron transfer during hydrogenase action.

Studies with various valence states of Mo and V have shown that in *A. vinelandii* (O strain) V can replace Mo for hydrogenase activity but only partly so in  $\text{N}_2$  fixation. This shows that hydrogenase activity and  $\text{N}_2$  fixation are not necessarily interdependent processes. Tungsten was found partly to replace Mo in  $\text{N}_2$  fixation in *A. chroococcum* strain 8003.

### Introduction

Iron, copper, zinc, manganese, molybdenum and boron are known to be essential for the growth of green plants. More recently the following additional nutrient requirements have been reported—chlorine for many higher plants,<sup>1</sup> sodium for a blue-green alga *Anabaena cylindrica*,<sup>2</sup> and vanadium for *Scenedesmus obliquus*.<sup>3</sup>

Two approaches have been used to establish if a mineral nutrient is essential for plant growth—(a) its omission from the diet resulting in a reduction in growth which can be remedied only by returning the nutrient to the plant in a readily available form, (b) establishing a rôle for the metal in intermediary metabolism. In practice the first method is often a prerequisite to the second.

The first method often entails the use of rigorous purification techniques to remove the metal from the basal culture solutions. At best these methods remove 99.99% of the metal and the residual amount, which can often be determined by isotope activation analysis, may be sufficient to support growth of the organism. The second method is direct but it is equally difficult to establish with certainty the sites and mode of action of metals in enzyme systems. Nevertheless the latter method has been used to good effect in recent years and is likely to be the method of the future in establishing the importance of other elements in intermediary metabolism.

The criteria for essentiality of a nutrient for plant growth, as set out by Arnon,<sup>4</sup> have served as a useful guide in plant nutrition studies. His concept that an element is essential

\* Read at Symposium on Trace Elements in Soils, Plants and Animals, organized by the Agriculture Group and Bristol Section, 10–12 April, 1957

for plant growth only when it cannot be replaced by another needs revision in the light of recent work. Thus the chlorine requirement of green plants can be replaced by bromine and to a lesser extent by iodine, but at much higher levels than for chlorine.<sup>1</sup> It is, however, reasonable to assume that chlorine is the halide most readily available when plants grow in their natural habitat and that chlorine is therefore an essential element. In certain *Azotobacter* strains V can partly replace Mo for the fixation of N<sub>2</sub>; thus both nutrients function in metabolism.<sup>5-7</sup> It is therefore better to redefine an essential element as one that functions in normal metabolism; then it may be termed a 'metabolism nutrient'.

In this paper a short account of recent work on the rôle of trace metals in the nitrogen metabolism of plants is presented.

### Nitrate assimilation

The assimilation of nitrate nitrogen by plants involves the reduction of nitrate to ammonia resulting in a valency change of the nitrogen atom from +5 to -3.

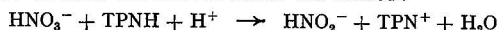
The importance of molybdenum in the reduction sequence was established when it was found that nitrate accumulated and free amino-acids were depleted in molybdenum-deficient tissue of fungi<sup>8</sup> and in green plants.<sup>9, 10</sup> These effects, however, are not specific for Mo, as a deficiency of Mn may give a similar result.<sup>11</sup> It was also established that the Mo requirements of fungi and higher plants were substantially reduced when ammonium ions were substituted for nitrate as the sole source of nitrogen.

### Reduction of aliphatic nitrogen compounds

#### Nitrate reductase

The attempts to demonstrate the presence of nitrate reductase enzyme in cell-free extracts of plants have been reviewed in detail by Nightingale,<sup>12</sup> Bürstrom,<sup>13</sup> Virtanen & Rautanen<sup>14</sup> and more recently by Nason<sup>15</sup> and Evans.<sup>16</sup>

An enzyme has been isolated from fungi and higher plants<sup>17</sup> which catalyses the reduction of nitrate to nitrite. This enzyme was shown to be a flavoprotein with flavin adenine dinucleotide (FAD) as the native flavin and reduced di- or triphosphopyridine nucleotide (DPNH or TPNH) as the electron donors. In *Neurospora* and *Aspergillus*<sup>17-19</sup> TPNH is the specific donor whereas in higher plants either nucleotide can function. Stoichiometric experiments showed that for each mole of TPNH oxidized one mole of nitrite was formed:



*Metal constituent.*—The enzyme was shown to be sensitive to metal inhibitors including cyanide, azide and hydroxyquinoline. Using the purification methods developed at Long Ashton<sup>8, 20</sup> it was possible to produce trace metal deficiencies in *Neurospora* including those of Mo and Cu. The results are shown in Table I.

A deficiency of Mo or of nitrate resulted in a significant reduction in the activity of nitrate reductase. The enzyme activity was increased appreciably when Cu, Zn or Fe was deficient. The low enzyme activity in felts grown with a deficiency of nitrogen was probably caused by a restricted synthesis of protein which would affect most enzymes.

Table I

Effect of nutrient deficiencies in *Neurospora crassa* on nitrate reductase in cell-free extracts of the felts (Wild type 146)

	Units of enzyme activity as μmoles of NO <sub>2</sub> ' formed per 10 min. per mg. of protein									
	+ Ca	- Ca	+ N	- N*	+ Mg	- Mg*	+ Fe	- Fe		
Growth, % of mycelium	100	16	100	8	100	20	100	0.7		
Nitrate reductase	26	38	43	5	49	41	29	55		
	+ Cu	- Cu	+ Zn	- Zn	+ Mn	- Mn	+ Mo	- Mo	+ Biotin	- Biotin
Growth, % of mycelium	100	40	100	28	100	30	100	29	100	15
Nitrate reductase	27	79	25	39	30	34	53	10	27	21

+ = felts grown in complete medium with the element  
 - = felts grown in absence or deficiency of the element

\* N and Mg were supplied at one-thirtieth and one-hundredth, respectively, of the level in the controls

The data in Table II indicate that enzyme activity depended on the presence of Mo when nitrate, nitrite or nitrate and ammonia were the sources of nitrogen. The enzyme was not formed when ammonia was the sole source of nitrogen and under these conditions the Mo requirement was considerably reduced but not eliminated. Arnon *et al.*,<sup>3</sup> have shown recently that in *Scenedesmus obliquus* the Mo requirement was nil when either ammonia or urea was substituted for nitrate as a nitrogen source. This result is, however, contrary to that obtained with fungi<sup>8, 9, 20</sup> and with higher plants.<sup>10</sup>

Table II

*Effect of molybdenum deficiency and nitrogen source on nitrate reductase in cell-free extracts of Neurospora crassa and Aspergillus niger*

Units of enzyme activity as $\mu$ moles of $\text{NO}_2^-$ formed per 10 min. per mg. of protein			Growth, %	Nitrate reductase
	Nitrogen source	Treatment		
<i>Neurospora</i> 146	Nitrate	+ Mo	100	21
		- Mo	29	5
	Nitrate and ammonia	+ Mo	100	17
		- Mo	30	10
	Nitrite	+ Mo	100	24
		- Mo	40	1
	Ammonia	+ Mo	100	2
		- Mo	70	0
<i>Neurospora</i> 5297a	Nitrate	+ Mo	100	11
		- Mo	32	1
	Nitrate and ammonia	+ Mo	100	19
		- Mo	24	1
	Nitrite	+ Mo	100	34
		- Mo	45	11
	Ammonia	+ Mo	100	3
		- Mo	65	1
<i>A. niger</i> (M strain)	Nitrate	+ Mo	100	39
		- Mo	5	2
	Nitrate and ammonia	+ Mo	100	42
		- Mo	75	20
	Ammonia	+ Mo	100	0
		- Mo	80	0

The evidence thus far showed that there is a connexion between Mo status and nitrate reductase activity in *Neurospora*, but whether the trace element was a constituent of the enzyme or whether it exerted its effect indirectly was yet to be determined.<sup>18</sup> It was shown that other enzymes requiring reduced pyridine nucleotides for their activity were unchanged in Mo-deficient felts (except for TPNH-dependent glutamic dehydrogenase, which was reduced). Further, the riboflavin content of normal felts was comparable with those deficient in Mo so that the flavin component of the enzyme was unlikely to be affected.<sup>18</sup>

The results of the following experiments showed Mo to be a constituent of nitrate reductase. An increase in the specific activity of the enzyme in various protein fractions of *Escherichia coli*, *Neurospora* and soya-bean respectively<sup>19, 21, 22</sup> was paralleled by an increase in their Mo contents. This effect was later confirmed by Evans<sup>23</sup> who showed that Mo labelled with <sup>99</sup>Mo accumulated in active nitrate reductase preparations from soya-bean. The other trace metals examined were not correlated with the enzymic activity.

Molybdenum was dialysed from the purified *Neurospora* enzyme by means of glutathione and cyanide, and incubation of the dialysed and washed preparation with either molybdenum trioxide or sodium molybdate (valency 6) restored its activity to about 80% of that of the undialysed enzyme. No other trace metal could be substituted for Mo in the reactivation process, as can be seen from Table III.

**Table III**

*Effect of metals on reactivation of cyanide-dialysed nitrate reductase from Neurospora crassa*

Unit of enzyme activity (as unit in Table I) per 0.05 ml. of enzyme

Experiment	Treatment 1	Treatment 2	Treatment 3	Treatment 4			
	Undialysed enzyme	Dialysed for 6 h. against 0.1M-phosphate + 10 <sup>-3</sup> M-glutathione*	Same as treatment 2 + 10 <sup>-3</sup> -cyanide	Same as treatment 3 then redialysed for 6 h. as in treatment 2			
I	18	14	4	4			
II	15	11	3	4			

After treatment 4, the salts indicated were added† and enzyme activity again determined

Experiment	MoO <sub>3</sub>	Na <sub>2</sub> MoO <sub>4</sub>	FeCl <sub>3</sub>	FeSO <sub>4</sub>	ZnSO <sub>4</sub>	MnSO <sub>4</sub>	NiCl <sub>2</sub>
I	13	14	5	6	4	3	4
II	12	11	4	4	3	3	2

	AgCl	Na <sub>2</sub> WO <sub>4</sub>	Na <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	Na <sub>2</sub> VO <sub>4</sub>	VO <sub>2</sub> (NO <sub>3</sub> ) <sub>2</sub>	CoSO <sub>4</sub>	Na <sub>2</sub> B <sub>2</sub> O <sub>4</sub>
I	4	4	5	3	4	3	4
II	3	3	4	3	4	3	4

\* Reagents freed from molybdenum

† 1 µg. of each metal added to 0.05 ml. of enzyme incubated for 10 min. at 25° before assay

Evans,<sup>18</sup> using a similar dialysis technique with enzyme from soya-bean, subsequently confirmed that a dialysed enzyme was reactivated by Mo and also slightly by Fe salts. The 'Fe effect' however may well have been caused by the presence of Mo as a contaminant since pure iron salts free from Mo do not appear to reactivate the enzyme.

*Mechanism of action.*—The rôle of Mo in the reduction sequence is now established thus: the reduction of FAD or FMN (flavin mononucleotides, riboflavin phosphate) by DPNH or TPNH is mediated equally well either by the purified enzyme or by the Mo-free enzyme. Only the normal enzyme was capable of transferring electrons from reduced flavin to nitrate but the addition of Mo to the Mo-free enzyme restored its capacity to do this.

It was then shown that sodium molybdate reduced with sodium hydrosulphite could act as an effective electron donor for the enzymic reduction of nitrate (Table IV).

This established that the element was undergoing a valency change during nitrate reductase action.

The valency states of molybdenum, viz., Mo<sup>6+</sup>, Mo<sup>5+</sup> and Mo<sup>3+</sup>, were separated by means of paper chromatography and it was shown<sup>24, 25</sup> that the most reduced valency state in the hydrosulphite-reduced molybdate was Mo<sup>5+</sup>. The effect of Mo<sup>5+</sup>, prepared free from the other valency states, as an electron donor for the enzymic reduction of nitrate is shown in Table V.

**Table IV**

*Hydrosulphite-treated molybdate as electron donor for enzymic nitrate reduction in Neurospora crassa*

µmoles of NO<sub>2</sub>' formed per 0.5 ml. of reaction mixture<sup>a</sup>

Experiment	TPNH, enzyme, FMN, KNO <sub>3</sub>	Reduced Mo, <sup>b</sup> enzyme, KNO <sub>3</sub>	Mo, enzyme, KNO <sub>3</sub>	Reduced blank, <sup>c</sup> enzyme, KNO <sub>3</sub>	Reduced Mo, <sup>b</sup> KNO <sub>3</sub>	Reduced Mo, <sup>b</sup> boiled enzyme, KNO <sub>3</sub>
1	24	19	7	3	1	4
2	30	25	9	8	2	6
3	20	15	2	2	1	2
4	23	20	4	2	2	3

<sup>a</sup> 0.30 ml. of enzyme containing approximately 500 µg. of protein, 0.24 ml. of TPNH (2 µmoles per ml.), 0.24 ml. of FMN (0.09 µmoles per ml.), 0.6 ml. of 0.1M-KNO<sub>3</sub>, and 0.5 ml. of 10<sup>-3</sup>M-Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, made up to 3.0 ml. with 0.1M-pyrophosphate buffer, pH 7.0. Reactions were started by adding the enzyme and nitrate to the other components of the reaction mixtures in evacuated modified Thunberg tubes. After 10-min. incubation at room temperature the mixtures were assayed for nitrite with sulphanilamide and *N*-(1-naphthyl)-ethylenediamine reagents.

<sup>b</sup> Reduced molybdate was prepared in the Thunberg tubes by adding approximately 2 mg. of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> to 0.5 ml. of 10<sup>-3</sup>M-Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O contained in the pyrophosphate buffer. H<sub>2</sub> gas was then bubbled through the solution for 10 min., followed by evacuation, incubation, and testing as indicated above.

<sup>c</sup> Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> and H<sub>2</sub> treatment as for 'reduced Mo' but without molybdate.

Table V

*Mo<sup>5+</sup> as an electron donor for the enzymic reduction of nitrate in Neurospora crassa*  
 mμmoles of NO<sub>2</sub><sup>-</sup> formed in the reaction mixture<sup>a</sup>

Experiment	TPNH, enzyme, FMN, KNO <sub>3</sub>	Mo <sup>5+</sup> , enzyme, KNO <sub>3</sub>	Mo <sup>5+</sup> , KNO <sub>3</sub>	Mo <sup>5+</sup> , boiled enzyme, KNO <sub>3</sub>	Mo <sup>6+</sup> , enzyme, KNO <sub>3</sub>
1	35.5	36	0	2	0
2	24	22	0	1	0

<sup>a</sup> 0.5 ml. of enzyme containing approximately 400 μg. of protein, 0.20 ml. of TPNH (2 μmoles/ml.), 0.20 ml. of FMN (0.09 μmoles/ml.), 0.6 ml. of 0.1M-KNO<sub>3</sub> and 50 μg. of Mo<sup>5+</sup> or Mo<sup>6+</sup> contained in 0.01 ml., checked for purity by paper chromatography. The reaction mixtures were made to 2 ml. with 0.1M-phosphate buffer, pH 7.5. After 10 min. at room temperature the mixtures were assayed for nitrite by using sulphanilamide and *N*-(1-naphthyl)ethylenediamine reagents.

Mo<sup>5+</sup> was as effective as TPNH as an electron donor for the enzymic reduction of nitrate. Mo<sup>6+</sup>, however, was without effect. In the absence of the enzyme or in the presence of the boiled enzyme, Mo<sup>5+</sup> did not reduce nitrate.

Mo<sup>3+</sup> was prepared by reducing acidified sodium molybdate with Zn dust. The excess Zn and residual Mo<sup>5+</sup> were removed from solution on a cellulose column. Mo<sup>3+</sup> thus prepared reduced nitrate non-enzymically whereas Mo<sup>5+</sup> or Mo<sup>6+</sup> did not. It is unlikely, however, that Mo<sup>3+</sup> is important in plant metabolism as it is readily oxidized to Mo<sup>5+</sup> in the presence of Mo<sup>6+</sup>, Fe<sup>3+</sup> or Cu<sup>2+</sup> which are invariably present in plant tissues. Mo<sup>4+</sup> is stable only in strong ethanol and it dismutates readily in the presence of a trace of water to give Mo<sup>3+</sup> and Mo<sup>5+</sup>; the Mo<sup>3+</sup> formed would then be readily oxidized to Mo<sup>5+</sup>.

Thus it appears that during the enzymic reduction of nitrate, Mo undergoes a one-electron change so that two Mo<sup>5+</sup> ions would be required to accept the two electrons from reduced flavin (FMNH<sub>2</sub> or FADH<sub>2</sub>).

The reduction of nitrate was followed by setting up a chemical model with Mo<sup>5+</sup> in the presence of nitrate. The spectra of Mo<sup>5+</sup> and Mo<sup>3+</sup> in an HCl-alcohol mixture showed a maximum at approx. 420 and an inflexion at 500 mμ, respectively. The oxidation of Mo<sup>3+</sup> and formation of Mo<sup>5+</sup> was followed at these two wavelengths (Mo<sup>5+</sup> gives very low optical density at 500 mμ), after addition of NO<sub>3</sub><sup>-</sup> to the Mo<sup>3+</sup> in the acid-alcohol solution. The results in Fig. 1 show that the oxidation of Mo<sup>3+</sup> is followed by a simultaneous appearance of Mo<sup>5+</sup> so that it is unlikely that an intermediate is formed in the process. Thus the mechanism is probably a transfer of two electrons from two Mo<sup>5+</sup> ions to the NO<sub>3</sub><sup>-</sup> anion, resulting in a rupture in the N-O bond.

The purified enzyme (over 80-fold) was shown recently to require phosphate for maximal activity<sup>26</sup> and is thus similar to other metallo-flavoproteins.<sup>27</sup> The phosphate requirement can be replaced completely by selenate, tellurate or arsenate and to a lesser extent by sulphate or silicate, each at a final concentration of 10<sup>-2</sup>M. Adenosine triphosphate (ATP) cannot substitute for inorganic phosphate and the enzyme is not inhibited by uncoupling reagents, e.g., dinitrophenol, aureomycin or sodium arsenite. It is likely, therefore, that one of the functions of phosphate in nitrate reductase is to bind the molybdate to the apoenzyme, as the replacement anions, which have similar atomic radii to that of phosphate (2.76Å), being within the range 2.4 to 2.80Å, also form complexes with molybdates.

*Distribution of the enzyme.*—Nitrate reductase occurs in bacteria, fungi and higher plants.<sup>16, 17, 19, 21, 22, 28</sup> In soya-bean inoculated with pure cultures of *Rhizobium japonicum*

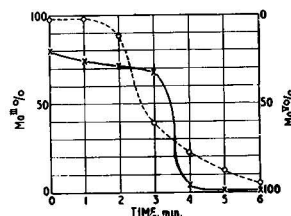


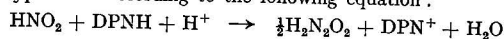
FIG. 1.—Rate of conversion of Mo<sup>3+</sup> to Mo<sup>5+</sup> followed at 420 mμ (for Mo<sup>3+</sup>) and at 500 mμ (for Mo<sup>5+</sup>)  
 Reaction mixture: 3 μg. of Mo<sup>3+</sup> in 2 ml. of 10N-HCl and 4 ml. of 98% v/v ethanol: reaction started by adding 0.1 ml. of N-KNO<sub>3</sub>

grown without combined nitrogen, an active nitrate reductase was found in the bacteria by Evans,<sup>28</sup> who has suggested that chemisorbed nitrogen, or an oxidized product, may induce the adaptive formation of nitrate reductase in the nodules. Although this is an attractive hypothesis, it is known that only traces of nitrate are required for the adaptive formation of the enzyme and in the author's experience it is difficult to eliminate nitrate-N completely from salts used in the culture solutions.

*Nitrite reductase*

This enzyme was first demonstrated in cell-free extracts of *Bacillus pyocyaneus* by Yamagata<sup>29</sup> and later in *Bacillus pumilis* by Taniguchi *et al.*,<sup>30</sup> who showed that nitrite was reduced to ammonia when leuco-methylene blue was the electron donor. It is unfortunate that much of the Japanese work is based on the use of dyes as electron carriers and that little attempt has been made to identify the native constituents of their systems.

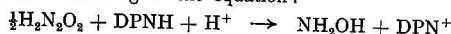
A nitrite reductase has been found in *Neurospora*<sup>31</sup> and soya-bean leaves which utilizes 3 moles of TPNH or DPNH for each mole of nitrite reduced to ammonia. The enzyme is a flavoprotein inhibited by metal binding agents. The soya-bean enzyme is stimulated by Mn but this effect is based on measurement of the extent of the oxidation of DPNH only and thus may well occur at any of the enzymic steps between NO<sub>2</sub> disappearance and ammonia production. Recent work in this laboratory has shown that nitrite is reduced by cell-free extracts of *Neurospora* to hyponitrite according to the following equation :



Preliminary results indicate that Cu and Fe are required for the enzymic reduction of NO<sub>2</sub><sup>-</sup> because Cu and Fe inhibitors, e.g., 2:2'-diquinolyl or diethylthiocarbamate, inhibit the enzyme and it is markedly less active in Cu- or Fe-deficient felts. The enzyme, prepared from felts deficient in Cu, is stimulated *in vitro* by the addition of the metal. There is a phosphorylation at this step because dinitrophenol and other uncouplers inhibit the enzyme.

*Hyponitrite reductase*

This enzyme, first identified in this laboratory,<sup>32</sup> requires DPNH as the electron donor and forms hydroxylamine according to the equation :



The results of the enzyme assay are shown in Table VI.

**Table VI**

*Hyponitrite reductase in Neurospora*

Experiment	(μmoles of NH <sub>3</sub> produced in 20 min./ml. enzyme)			Specific activity of complete system, μmoles of NH <sub>3</sub> /20 min./mg. of protein
	Complete system	Without DPNH	Without enzyme	
1	288	48	0	34.7
2	237	49	0	28.5
3	411	51	0	28.3

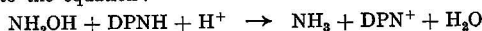
*Complete system*: Outer cell of Conway unit contained 0.15 ml. of 0.2M-pyrophosphate (pH 7.5); 0.1 ml. of 4 × 10<sup>-3</sup>M-Na<sub>2</sub>N<sub>2</sub>O<sub>2</sub>; 0.05 ml. of boiled pig heart, 0.2 ml. of enzyme (8.3 mg./ml.), 0.1 ml. of 10<sup>-2</sup>M-DPNH.  
 Inner cell: 1.5 ml. of 0.01N-HCl.  
 All reagents (except enzyme) were boiled before assay to remove ammonia.  
 The system was incubated for 20 min., then 1 ml. of saturated K<sub>2</sub>CO<sub>3</sub> was added to the outer cell followed by further incubation for 1½ h. Then 0.4 ml. of inner solution was tested for NH<sub>3</sub> by the phenol-hypochlorite method.

This enzyme is inhibited also by metal-chelating agents, including 8-hydroxyquinoline, cyanide and 'tetrabase' (tetramethyldiaminodiphenylmethane), and by uncoupling reagents, e.g., dinitrophenol and aureomycin. Thus this step appears also to be linked to an ATP energy-conserving system. The enzyme is reduced in Cu- or Fe-deficient felts.

*Hydroxylamine reductase*

Hydroxylamine reductase was first observed by Japanese workers<sup>29, 30</sup> in bacteria with the aid of artificial dye carriers. As in nitrite reductase the native constituents of this system

have not been characterized. Subsequently this enzyme, found in *Neurospora* and higher plants, was shown by Zucker & Nason<sup>33</sup> to have properties similar to those of nitrite reductase, but the two enzymes are distinct. Hydroxylamine reductase is flavin-dependent and is inhibited by metal-chelating agents. The soya-bean enzyme appears to be stimulated by Mn, based on ammonia production and DPNH oxidation. The stoichiometric measurements indicate that hydroxylamine reductase catalyses the conversion of one mole of NH<sub>2</sub>OH to one mole of NH<sub>3</sub> according to the equation:



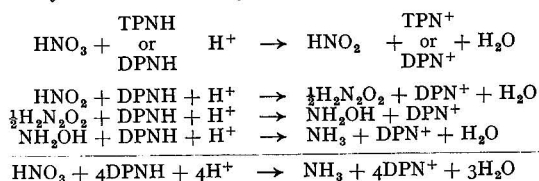
Recent work in this laboratory has shown that extracts from Mn-deficient felts of *Neurospora* failed to catalyse this reaction, whereas those from felts deficient in other trace metals showed maximal enzyme activity. This enzyme is unaffected by uncoupling reagents.

Klausmeyer & Bard<sup>34</sup> have found ammonia dehydrogenase in *Bacillus subtilis* which reversibly catalyses the formation of ammonia and DPN from DPNH and hydroxylamine.

The mechanism and pathway of reduction of nitrate to ammonia in plants is still not fully known but recent work indicates that the following enzymic steps, in the aliphatic series, occur in *Neurospora* and in green plants:

Electron donor	TPNH or DPNH			
Co-factor	FAD			
Metal constituent	Mo	Cu, Fe	Cu, Fe	Mn
Valency state of N	+ 5 <sup>2e</sup> + 3 NO <sub>3</sub> → NO <sub>2</sub>	+ 3 + 1 NO <sub>2</sub> → N <sub>2</sub> O <sub>2</sub>	+ 1 - 1 N <sub>2</sub> O <sub>2</sub> → NH <sub>2</sub> OH	- 1 - 3 NH <sub>2</sub> OH → NH <sub>3</sub>
Phosphorylation	—	P	P	—

The stoichiometry of the reactions may be summarized as follows:



The basic assumption in this sequence is that two electron changes are involved in each step which implies an intermediate having a valency state for N of + 1. This is now supported by direct experimental proof of the existence of hyponitrite reductase in plants.<sup>32</sup>

#### Reduction of organo-nitro compounds

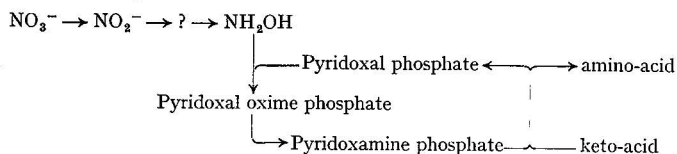
An alternative or simultaneous mechanism for reducing nitrate may involve organo-nitro compounds. It is known that living tissues reduce these compounds. Thus Lipschitz<sup>35</sup> showed that *m*-dinitrobenzene was reduced to *m*-nitrophenylhydroxylamine by muscle tissue and Saz & Slie<sup>36</sup> found that extracts of *Escherichia coli* reduced nitro-groups of *p*-nitrobenzoic acid and other related nitro compounds to the corresponding arylamines. In *Neurospora*<sup>37</sup> an adaptive enzyme has been identified which reduces *m*-dinitrobenzene to *m*-nitroaniline in the presence of DPNH or TPNH, via the intermediates *m*-nitroso-nitrobenzene and *m*-nitrophenylhydroxylamine.

This enzyme is a flavoprotein and is sensitive to metal inhibitors but the metal involved has not been identified. Nitrophenylhydroxylamine has been identified as an intermediate in the reductive sequence but the enzyme is non-specific and can reduce a range of nitro aryl



compounds. It is significant that this enzyme is found only when *m*-dinitrobenzene is present in the basal culture media whereas the enzymes in the aliphatic series occur when nitrate or nitrite is the N source. Thus it is more likely that the aliphatic reductive series is the usual way of assimilating nitrate.

Further evidence for the organic pathway has been presented by Silver & McElroy<sup>38</sup> using mutants of *Neurospora* blocked at various points in the reductive sequence. Cell-free extracts of the mutants unable to grow on nitrate or nitrite were found, however, to reduce nitrite through hydroxylamine to ammonia. These authors found that these mutants required the addition of pyridoxine to the media before they could grow on nitrite but when ammonia was the sole N source the vitamin was not required. Pyridoxine-requiring mutants were also found to be low in nitrite reductase when the vitamin was deficient. Silver & McElroy suggest the following sequence to explain the results:



Although this is a very interesting hypothesis, further work is required to prove it. It would now be interesting to assay the mutants for hyponitrite reductase and to use the revised assay for nitrite reductase proposed by Medina & Nicholas<sup>39</sup> who found the old method to be in error.

#### Nitrogen fixation

Trace metals are known to be essential for the fixation of atmospheric nitrogen in free living bacteria, e.g., *Azotobacter* and *Clostridia*, and in root nodule bacteria, e.g., *Rhizobia*.<sup>40</sup> Thus Bortels<sup>5</sup> and Horner *et al.*<sup>6</sup> demonstrated a molybdenum requirement for nitrogen fixation in *Azotobacter* which could be partially replaced by vanadium. More recently Virtanen<sup>7</sup> has confirmed these effects in *Azotobacter vinelandii* and *Clostridium butylicum*.

In this laboratory, using culture solutions specially freed from trace metals, the micro-nutrient requirements in *Azotobacter* were found to vary with the strains used. Iron deficiency, which is readily produced in all the strains examined, results in reduction in growth and nitrogen fixation. Mo deficiency gives restricted growth in a few strains only, because the requirement for this metal appears to be markedly less than that for Fe. In *Azotobacter chroococcum* 8003, vanadium or tungsten can partially replace Mo for growth, whilst in *Clostridium pasteurianum* W-5, Fe is required in relatively larger amounts than Mo for growth and nitrogen fixation.

#### Hydrogenase

Stephenson & Stickland<sup>41</sup> were the first to show that several heterotrophic species of bacteria could reversibly activate hydrogen, the enzyme responsible being called hydrogenase. Lee, Wilson & Wilson<sup>42</sup> showed that the hydrogenase activity in *Azotobacter* was correlated with the functioning of the nitrogen-fixing system. Cells grown with combined nitrogen had considerably less enzyme activity than those given nitrogen only. Koffler & Wilson<sup>43</sup> have suggested that nitrogen adsorbed by a (non-characterized) nitrogenase enzyme is reduced by the hydrogenase system. This hypothesis, however, is not compatible with the following facts: (a) hydrogenase has not been detected in root nodule bacteria which actively fix nitrogen, (b) the enzyme is present in bacteria which require combined nitrogen for growth, e.g., *Escherichia coli*, and (c) the enzyme may be active even when nitrogen fixation is suboptimal.

Recent experiments by Shug, Hamilton & Wilson<sup>44</sup> with cell-free preparations of hydrogenase from *Clostridium pasteurianum* indicate that the enzyme is a molybdo-flavoprotein. Both FAD and Mo were required to reactivate the purified enzyme. Waring & Werkman<sup>45</sup> have shown that Fe is required for the formation of hydrogenase in *Aerobacter aerogenes*.

In our experiments (Table VII), Fe deficiency drastically reduced the enzyme, measured in whole cells and in cell-free extracts of *Azotobacter vinelandii* O strain and also in those of *Clostridium pasteurianum* W-5. A deficiency of Mo, however, had no effect on the *Azotobacter* enzyme and reduced its activity only slightly in *Cl. pasteurianum* W-5. Thus, Fe appears to be as essential for hydrogenase activity in the two bacteria as it is for their growth. The enzyme

**Table VII**  
*Hydrogenase activity in Azotobacter and in Clostridium*  
( $\text{QH}_2$  per mg. of nitrogen fixed)

Organism	<i>Azotobacter vinelandii</i>			<i>Clostridium pasteurianum</i>		
	Full culture	Deficiency of		Full culture	Deficiency of	
Nutrient solution		Fe	Mo		Fe	Mo
<i>Hydrogenase assay</i>						
Whole cells	4620	1150	4150	59,400	16,200	48,100
	4370	570	3800			
	2640	280	2400	31,000	7000	26,000

prepared from Fe-deficient bacteria could not be reactivated by adding to it  $\text{Fe}^{3+}$ ,  $\text{Fe}^{2+}$ , Fe Versenate, cytochrome *c*, or a boiled extract of normal cells.

In *Azotobacter* (O strain) hydrogenase is associated with cell particles, as only 5% of the total enzyme activity was present in extracts prepared in a Hughes press and centrifuged at 15,000g, whereas in *Clostridium pasteurianum* W-5 about 60% of the total activity was in the cell-free preparation. In tests with radioactive tracers, the distribution of  $^{59}\text{Fe}$  in the cell particles of *Azotobacter*, separated by differential centrifugation, followed closely that of hydrogenase activity, while both  $^{59}\text{Fe}$  and  $^{99}\text{Mo}$  were removed slowly from homogenates of *Azotobacter* dialysed against a variety of chelating agents, indicating that they were tightly bound to the cell proteins.<sup>46</sup>

The effects of adding Mo and V in various valency states to culture solutions of *A. vinelandii* O strain on nitrogen fixation and hydrogenase activity are given in Table VIII.

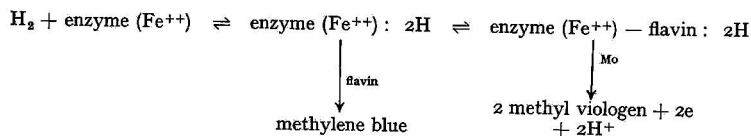
**Table VIII**  
*Effect of valency states of molybdenum and vanadium on the Mo and V uptakes, nitrogen fixation and hydrogenase activity of Azotobacter vinelandii (O strain)*  
(5-l. cultures containing 1 mg. of Mo or V)

Valency state of molybdenum or vanadium	Mo <sup>VI</sup>	Mo <sup>V</sup>	Mo <sup>III</sup>	V <sup>V</sup>	V <sup>IV</sup>	V <sup>III</sup>	Deficiency of both Mo and V
Total uptake of metal ( $\mu\text{g.}$ )	579	558	300	206	271	966	not detected
Total nitrogen fixed in cells (mg.)	318	265	180	160	135	83	83
Hydrogenase ( $\mu\text{l. of H}_2$ fixed per mg. protein per h.)	920	840	1270	1130	955	1010	877

In the absence of Mo and V, nitrogen fixation only was markedly reduced. Although in all the cases there was high hydrogenase activity, nitrogen fixation was greater when  $\text{Mo}^{6+}$  or  $\text{Mo}^{5+}$  was supplied. The cells given  $\text{Mo}^{3+}$  or  $\text{V}^{3+}$  had an active hydrogenase yet fixed the smallest amount of nitrogen. It appears that all the Mo or V valencies are equally effective in producing active hydrogenase but the cells given V fixed less nitrogen than did those given Mo. Thus in *Azotobacter* an active hydrogenase is not always accompanied by an effective nitrogen fixation.

Peck, San Pietro & Gest<sup>47</sup> have recently proposed the following mode of action for hydrogenase :

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According to this scheme, molecular hydrogen is activated by a ferrous enzyme to form a reduced enzyme complex which can be oxidized through a flavin component to methylene blue or similar two-electron acceptors. Alternatively Mo could combine with the enzyme and the reduced flavin could be reoxidized by one electron acceptor such as viologen dyes or cytochrome *c*. The reduction of viologens by hydrogen and the formation of hydrogen from reduced methyl dye would require flavin and the two metals. If Mo was absent, then hydrogenase would react only with methylene blue. In support of the above scheme it has been shown that in hydrogenase from *Clostridium pasteurianum*, Mo is required for the reduction of cytochrome *c* but not of methylene blue. The precise rôles of the metals in hydrogenase, however, remain obscure. By analogy with the nitrate reductase system,<sup>24, 25</sup> it is likely that the two metals undergo valency changes during hydrogenase action.

References

- <sup>1</sup> Stout, P. R., Johnson, C. M., Carlton, A. B., & Broyer, T. C., *Plant Physiol.*, 1954, **29**, 526  
<sup>2</sup> Allen, M. B., & Arnon, D. I., *Physiol. Plant.*, 1955, **8**, 653  
<sup>3</sup> Arnon, D. I., Ichioka, S. P., Wessel, G., Fujiwara, A., & Woolley, J. T., *Physiol. Plant.*, 1955, **8**, 538  
<sup>4</sup> Arnon, D. I., in 'Trace Elements in Plant Physiology', 1950, p. 31 (Waltham, Mass.: Chronica Botanica Co.)  
<sup>5</sup> Bortels, H., *Arch. Mikrobiol.*, 1930, **1**, 333  
<sup>6</sup> Horner, C. K., Burk, D., Allison, F. E., & Sherman, M. S., *J. agric. Res.*, 1942, **65**, 173  
<sup>7</sup> Virtanen, A. I., in 'Conférences et Rapports', 3rd Int. Congr. Biochem., Brussels, ed. Liebecq, C., 1956, p. 425  
<sup>8</sup> Nicholas, D. J. D., *J. Sci. Fd Agric.*, 1950, **1**, 339  
<sup>9</sup> Mulder, E. C., *Plant & Soil*, 1948, **1**, 94  
<sup>10</sup> Hewitt, E. J., & Bolle-Jones, E. W., *J. hort. Sci.*, 1952, **27**, 245  
<sup>11</sup> Leeper, G. W., *J. Aust. Inst. agric. Sci.*, 1941, **7**, 161  
<sup>12</sup> Nightingale, G. T., *Bot. Rev.*, 1937, **3**, 85; 1948, **14**, 185  
<sup>13</sup> Børstrem, H., *Planta*, 1939, **30**, 129  
<sup>14</sup> Virtanen, A. I., & Rautanen, N., in 'The Enzymes', eds. Sumner, J. B., & Nyrbäch, K., Vol. II, p. 1089 (New York: Academic Press)  
<sup>15</sup> Nason, A., in 'Inorganic Nitrogen Metabolism', eds. McElroy, W. D., & Glass, B., 1956, p. 109 (Baltimore: Johns Hopkins Univ. Press)  
<sup>16</sup> Evans, H. J., *Soil Sci.*, 1956, **81**, 199  
<sup>17</sup> Nason, A., & Evans, H. J., *J. biol. Chem.*, 1953, **202**, 655  
<sup>18</sup> Nicholas, D. J. D., Nason, A., & McElroy, W. D., *J. biol. Chem.*, 1954, **207**, 341  
<sup>19</sup> Nicholas, D. J. D., & Nason, A., *J. biol. Chem.*, 1954, **207**, 353; **211**, 183  
<sup>20</sup> Nicholas, D. J. D., *Analyst*, 1952, **77**, 629  
<sup>21</sup> Nicholas, D. J. D., & Nason, A., *J. Bact.*, 1955, **69**, 580  
<sup>22</sup> Nicholas, D. J. D., & Nason, A., *Plant Physiol.*, 1955, **30**, 135  
<sup>23</sup> Evans, H. J., *Science*, 1955, **122**, 922  
<sup>24</sup> Nicholas, D. J. D., & Stevens, H. M., *Nature, Lond.*, 1955, **176**, 1066  
<sup>25</sup> Nicholas, D. J. D., & Stevens, H. M., in 'Inorganic Nitrogen Metabolism', eds. McElroy, W. D., & Glass, B., 1956, p. 178 (Baltimore: Johns Hopkins Univ. Press)  
<sup>26</sup> Nicholas, D. J. D., & Scawin, J. H., *Nature, Lond.*, 1956, **178**, 1474  
<sup>27</sup> Mahler, H. R., in 'Conférences et Rapports', 3rd Int. Congr. Biochem., Brussels, ed. Liebecq, C., 1956, p. 252  
<sup>28</sup> Evans, H. J., *Plant Physiol.*, 1954, **29**, 298  
<sup>29</sup> Yamagata, S., *Acta phytochim., Tokyo*, 1939, **11**, 145  
<sup>30</sup> Taniguchi, S., Sato, R., & Egami, F., in 'Inorganic Nitrogen Metabolism', eds. McElroy, W. D., & Glass, B., 1956, p. 87 (Baltimore: Johns Hopkins Univ. Press)  
<sup>31</sup> Nason, A., Abraham, R. G., & Averbach, B. C., *Biochem. biophys. Acta*, 1954, **15**, 159  
<sup>32</sup> Medina, A., & Nicholas, D. J. D., *Nature, Lond.*, 1957, **179**, 533  
<sup>33</sup> Zucker, M., & Nason, A., *J. biol. Chem.*, 1955, **213**, 463  
<sup>34</sup> Klausmeyer, R., & Bard, R., *J. Bact.*, 1954, **68**, 129  
<sup>35</sup> Lipschitz, W., *Hoppe-Seyl. Z.*, 1920, **109**, 189  
<sup>36</sup> Saz, A. K., & Shie, R. B., *Arch. Biochem. Biophys.*, 1954, **51**, 5  
<sup>37</sup> Zucker, M., & Nason, A., in 'Methods in Enzymology', eds. Colowick, S. P., & Kaplan, N. O., 1956, Vol. II, p. 406 (New York: Academic Press)  
<sup>38</sup> Silver, W., & McElroy, W. D., *Arch. Biochem. Biophys.*, 1954, **51**, 379  
<sup>39</sup> Medina, A., & Nicholas, D. J. D., *Biochim. biophys. Acta*, 1957, **23**, 440  
<sup>40</sup> Burris, R. H., in 'Inorganic Nitrogen Metabolism', eds. McElroy, W. D., & Glass, B., 1956, p. 316 (Baltimore: Johns Hopkins Univ. Press)  
<sup>41</sup> Stephenson, M., & Strickland, L. H., *Biochem. J.*, 1932, **26**, 712  
<sup>42</sup> Lee, S. B., Wilson, T. B., & Wilson, P. W., *J. biol. Chem.*, 1942, **144**, 273  
<sup>43</sup> Koffler, H., & Wilson, P. W., in 'Bacterial Physiology', eds. Werkman, C. W., & Wilson, P. W., 1951, p. 52 (New York: Academic Press)  
<sup>44</sup> Shug, A. L., Wilson, P. W., Green, D. E., & Mahler, H. R., *J. Amer. chem. Soc.*, 1954, **76**, 3355  
<sup>45</sup> Waring, W. S., & Werkman, C. H., *Arch. Biochem. Biophys.*, 1944, **4**, 75  
<sup>46</sup> Nicholas, D. J. D., unpublished results  
<sup>47</sup> Peck, D. H., jun., San Pietro, A., & Gest, H., *Proc. nat. Acad. Sci., Wash.*, 1956, **42**, 13

**Discussion**

*Dr. E. C. Owen* (Hannah Dairy Research Institute) : Is FAD comparable with molybdenum in being removable and replaceable with loss and with regain of activity of nitrate reductase ?

*Dr. Nicholas* : Yes, the FAD can be replaced.

*Dr. Owen* : Is the molybdenum valency state as important in xanthine oxidase as in nitrate reductase ? Is this perhaps why molybdenum activation of xanthine oxidase has not been demonstrated ?

*Dr. Nicholas* : Molybdenum can be taken away from xanthine oxidase which can be reactivated provided either silicate or phosphate is present to bind the molybdenum, but as yet the valency states of molybdenum in the xanthine oxidase system have not been determined.

*Dr. Owen* : Is the nitrate reductase in cytoplasm or in the chloroplasts ? *Dr. H. E. Davenport* (Long Ashton) : There is no evidence for the association of nitrate reductase with chloroplasts. *Dr. Hewitt* : Light is essential for nitrate reductase activity in higher plants.

*Dr. Owen* : Do roots contain nitrate reductase ?

*Dr. Hewitt* : As far as I know nitrite reductase has been detected but not nitrate reductase. *Professor Wallace* : In early work it has been stated that nitrate itself cannot be detected in the roots of apple trees.

*Dr. J. V. Possingham* (Oxford) : Is it possible that nitrite and hyponitrite reductases might be identical ?

*Dr. Nicholas* : This is unlikely since zinc deficiency reduces the activity of nitrite reductase but has no effect on hyponitrite reductase.

*Dr. J. Tinsley* (Reading) : In Table III is the activity of the nitrate reductase following removal of molybdenum by cyanide treatment still measurable in the presence of various metal cations ? Can this be attributed to incomplete removal of molybdenum from the original enzyme ?

*Dr. Nicholas* : A maximum of only 80% of the molybdenum can be removed ; similarly replacement can only reach a maximum of 85%.

*Dr. H. E. Davenport* (Long Ashton) : On what evidence does *Dr. Nicholas* postulate phosphorylation at the nitrite and hyponitrite stages ?

*Dr. Nicholas* : On the grounds that uncoupling reagents inhibit these two stages but not the steps nitrate to nitrite and hydroxylamine to ammonia. *Dr. Davenport* : The energy changes involved in these steps might prove of interest.

## TRACE ELEMENTS AND CROP PRODUCTION IN THE TROPICS\*

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### Introduction

The main land masses lying in the tropics are parts of South America, Africa, India, Indo-China and Australia, while numerous islands between Malaya and Australia make up a much smaller area. Over the major portion of the areas the natural vegetation is forest, and most of the crops produced are destined to provide subsistence for the indigenous population. In the past the simplest agricultural systems have been effective and in some areas are still working satisfactorily. Land subject to freshwater flooding is used for rice production, either as a continuous monoculture or alternating with a fallow of sorts. On the dry land, forest is felled, burned and cleared, and the land cropped for a limited period. When yields fall, fresh clearings are made and the old clearings abandoned to return to forest. This is called the 'Shifting System' and formerly the shifts were unsystematic.

Force of circumstances has, however, in many areas driven the population to adopt more systematic moves. Where increasing population has led to restriction in the scope for movement, villages have become stabilized within recognized boundaries. Shifting now takes place within these boundaries and becomes virtually a rotation system in which a portion of the land is cultivated for a limited period and abandoned to forest long enough to recover before being brought into cultivation again. Roughly one quarter of the land can be under crop at any one time and as long as this proportion is not exceeded the system can be maintained indefinitely. In the Belgian Congo the systematic shifting system goes under the name of 'Corridor System'. Strips of definite width and orientation are cleared, cultivated for 2½ years and allowed to return to forest condition, which takes about 8 years. The apparent preponderance of uncultivated land in many tropical areas is not evidence of a large surplus of land over and above immediate needs. The land is generally in a recovery stage, vital to the success of the agricultural system in use, and failure to realize this has led to economic and political difficulties.

The Shifting System cannot continue indefinitely to support increasing population and higher living standards. A point is reached where the natural productivity is less than the demand. If this higher demand is met by increasing the cultivated area and reducing the area of recovering land, the safe proportion of land under crop may be exceeded. Recovery may fall short of forest and be terminated in the 'bush' stage. This leads to a reduced yield or shorter cropping period. In either case greater inroads are made into the recovering fallows, aggravating the situation. The Shifting System is not capable of development beyond a certain point and any attempt to do so must lead to breakdown and soil exhaustion. There are already several instances of large-scale re-settlement made necessary by the failure of the Shifting System.

If present trends are maintained, some alternative system capable of giving higher, sustained yields without damaging the soil must be found before the breakdown of the Shifting System becomes widespread. Engledow<sup>1</sup> holds that a shifting system is adopted only because other systems cannot maintain the fertility of the land with continuous cultivation. Systems aimed at maintaining soil fertility, involving mixed farming or green manuring, have been tried without any great success. Too often the return per unit of area is insufficient to carry the human dependants.

Reliable figures for crop yields are not available for much of the tropics, but figures for Africa show that in general, and for cereals in particular, the average yields are lower than in Europe or North America. This is true of farms and plantations run by Europeans as well as of the more primitive indigenous agriculture. Pests and diseases, together with poor climatic conditions, are sometimes responsible for poor yields but do not provide a general answer for

\* Read at Symposium on Trace Elements in Soils, Plants and Animals, organized by the Agriculture Group and Bristol Section, 10-12 April, 1957

low productivity. Deficiencies of essential mineral nutrients, either inherent in the soil or arising during cultivation, must be regarded as a likely cause of low yields, and as a possible explanation of the failure of the land to support continued cultivation.

#### The use of fertilizers on tropical soils

Increasing attention is being given to the use of fertilizers in tropical agriculture. Following usual European practice, attention has been focused on nitrogen, phosphorus and potassium, singly or in combination. This preoccupation with N, P and K has led to some misinterpretations of results and has tended to restrict thought and experimentation. Spectacular responses have been obtained with NPK fertilizers in some trials, but it is rare to find the yields reaching maximal values, even with massive applications of fertilizer. Very often quite small doses give as good results as much larger amounts. In Senegal, for instance, it has been found that groundnuts give a response to 150 kg. per hectare of a compound NPK fertilizer which is not increased even though the rate of application be quadrupled. Moreover, the higher yields obtained with fertilizer are still short of normal yields in other parts of the world where groundnuts are grown commercially.

In the Gambia, between 1924 and 1928, the Department of Agriculture conducted fertilizer trials on land cleared after 8 years' fallow. Eight major element fertilizers were used, including some mixtures, but in no treatment was there an increase in yield of groundnuts over the control. Furthermore, after the second year all yields fell by some 40%, irrespective of treatment. This fall is a common feature of the cultivation cycle used by the local population. Clearly some factor or factors not contained in the trials had a major effect on soil fertility.

Although the pH of tropical soils is often markedly acid, the effect of even moderate applications of lime appears to be more frequently harmful than beneficial. This may be due to the low buffer capacity of many tropical soils permitting a rise in pH sufficient to immobilize some of the essential trace elements. Coupled with the limitation or absence of response to fertilizers containing only major elements, this information leads one to conclude that trace-element deficiencies are at least a probability in some tropical soils.

#### Importance of trace elements

In recent years the remaining 9 or 10 elements other than N, P and K known to be essential for plant growth have begun to receive attention. Some responses to trace elements have been recorded from tropical areas. Ferrand, Bachy & Ollagnier<sup>2</sup> reported increased fruit production in oil palms due to Zn, Cu, Mn, B and Fe applied along with a NPK mixture which had itself a positive effect. Joshi & Joshi<sup>3</sup> record rice yields being increased by one third following the application of copper sulphate, while the application of copper plus manure gave a 77% increase over a manured crop yield. As a result of a questionnaire circulated to workers in Africa, Schutte<sup>4</sup> was able to report trace-element deficiencies in practically every territory in tropical Africa. Deficiencies have been reported also from other tropical territories. The distribution of the deficiencies so far recorded can be seen in Table I.

How great is the effect of trace-element deficiencies on the yields of the respective crops it is not possible to say. Experimentally the situation must be regarded as one of considerable complexity, in which a response to any element might appear as a high-order interaction and not as a primary effect at all. In a simple pot trial in the Gambia in 1950, using field soil and a grain sorghum as test plant, it was found that the application of a mineral nutrient solution containing 11 of the essential elements resulted in an increase in dry weight of the test plants of 74%. Applying the major elements separately without the trace elements gave an increased growth of only 12%, while the trace elements alone gave only 15% increase. This shows clearly that major element responses can be limited drastically by trace-element deficiencies and *vice versa*.

Although of little diagnostic value, the experiment showed that there were at least two elements whose deficiency caused limitation of growth and that unless both elements were supplied simultaneously worthwhile responses could not be obtained. One cannot of course exclude the possibility of a greater number of deficiencies. If the possibility that two or more deficiencies may exist concurrently is admitted—and in an unknown situation there is no

Table I

Trace-element deficiencies reported in the tropics					
Deficiency	Crop	Territory	Deficiency	Crop	Territory
Boron	Oil palm	Belgian Congo, French Equatorial Africa	Molybdenum	Legumes	Madagascar, Senegal, Gambia
	Coffee	French West Africa		Zinc	Oil palm
	Groundnuts	Gambia	Pineapples		Cameroons, Senegal
	Citrus	Rhodesia, S. Africa	Citrus		French Equatorial Africa, Uganda, Rhodesia, Zanzibar, Australia, S. Africa
	Vegetables	Rhodesia, Gambia, S. Africa	Iron	Bananas	French Equatorial Africa
Copper	Oil palm	Belgian Congo, French Equatorial Africa		Cocoa	Nigeria
	Rice	India		Wheat	Australia
	Groundnuts	Senegal, Gambia		Tea	Nyasaland
	Cereals	Kenya, Australia, S. Africa		Coffee	Ivory Coast
	Citrus	Rhodesia, Uganda, S. Africa	Groundnuts	Gold Coast	
Manganese	Cocoa	Zanzibar	Cashew	Tanganyika, Zanzibar	
	Oil palm	French Equatorial Africa	Citrus, cococa	Zanzibar	
	Wheat	Tanganyika	Oil palm	French Equatorial Africa	
	Tomatoes	Tanganyika			
	Citrus and other fruit	Rhodesia, S. Africa			

*a priori* reason to exclude such a possibility—the absence of a response to a particular element has no particular significance. Lack of response might be due either to another simultaneous deficiency, or to the fact that the element supplied was not deficient. The interpretation is ambiguous. In a trial under these conditions only a positive response has any constructive value, and one is faced with the necessity of testing a large number of mixtures of increasing complexity, looking for relatively high-order interactions. For diagnostic purposes such a procedure is too unwieldy for general use.

**Diagnosis of mineral deficiencies by the subtractive method**

An alternative to the usual procedure was sought which could make diagnosis of deficiencies easier. It was thought that if a reasonably balanced complete fertilizer, containing all known essential elements, were used as a standard, comparisons might be possible between the response to the complete fertilizer and responses to deficient mixtures prepared by omitting each element in turn from the complete mixture. The technique of preparing the mixtures has been worked out by Hewitt<sup>5</sup> and used by him to produce visual deficiency symptoms for the essential elements in a variety of test plants. This method, of comparing the effect of omission of any element from a complete mixture with the response when the element is not omitted, might be called the 'subtractive' method.

The method was tried out in pots in the Gambia in 1953-54 using field soils taken from plots on the Yundum Experimental Farm. The complete nutrient mixture used was one which has given good results with many crops on purified sand. It had the following composition :

KNO <sub>3</sub>	10.1 g.	MnSO <sub>4</sub> .4H <sub>2</sub> O	44.6 mg.
Ca(NO <sub>3</sub> ) <sub>2</sub>	16.4 g.	CuSO <sub>4</sub> .5H <sub>2</sub> O	5.0 mg.
NaH <sub>2</sub> PO <sub>4</sub> .2H <sub>2</sub> O	4.2 g.	ZnSO <sub>4</sub> .7H <sub>2</sub> O	5.8 mg.
MgSO <sub>4</sub> .7H <sub>2</sub> O	7.4 g.	H <sub>3</sub> BO <sub>3</sub>	37.2 mg.
Ferric citrate	0.5 g.	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> .4H <sub>2</sub> O	0.7 mg.

These amounts were dissolved in 20 l. of water for application at the rate of 1 l. per day for each 11-in. pot of adult plants. Later it was found that the applications could be reduced to alternate days, or even every third day, without detriment to growth, water being used in the intervening periods. The chemicals were purified as described by Hewitt and water of sufficient purity was used throughout. Controls which received only water were also set up.

Table II contains some of the weights recorded during several trials. The figures show that, compared with the controls, the fertilizer mixtures mostly produced an increase in weight.

Table II

Weight (g.) of various plants grown with complete fertilizer and fertilizer deficient in various elements

Plant	..	Tomato	Tomato	Maize	Cabbage	Groundnut	Lettuce
Part weighed	..	plant	fruit	plant	plant	plant	plant
Fresh or dry	..	dry	fresh	dry	fresh	dry	fresh
Age, days	..	60	153	60	120	150	120
Treatment							
Complete		41.0	1046	80.0	970	131	522.0
Omit N		2.3	15	5.3	17	162	
Omit P		1.0	0	5.6	15	0	
Omit K		23.0	255	25.7	463	82	
Omit Ca		45.0	1176	49.2	932	58	
Omit Mg		37.5	886	57.0	1272	72	
Omit S		48.5	727			65	
Omit B		39.0	1091	84.2		116	
Omit Mn		44.5	1111	59.2	664	114	
Omit Cu			1152	41.5	1015	146	
Omit Zn			911	51.5	956	155	
Omit Mo			1059		678	155	88.6
Control		1.0	0	4.6	3.7	62	4.2

The results with the fertilizer from which P was omitted are, however, sometimes inferior to those for the control. This is thought to be due to a reduction in the already low level of available P, through bacterial action, which occurs when the 'Omit P' mixture has been applied. Leaching did not take place. Judging not only by the weights of plant or crop produced, but also by the appearance of visual deficiency symptoms on the plants, it was concluded that the method indicated the probable existence simultaneously of several mineral deficiencies capable of restricting yield.

The omission of N or P has the most marked effects, followed by those with omission of K or S. The results vary with the test plant, groundnuts being unaffected by the omission of N in the presence of all other elements. As far as the trace elements are concerned, the omission of either Mn, Cu or Zn affects the growth of maize. The omission of Mn or Mo affects the growth of cabbage, while lack of Mo has a very marked effect on lettuce. All the soils tested indicated the probable presence of multiple deficiencies involving four or more elements, the omission of which from a fertilizer mixture could reduce the effectiveness of the fertilizer by 50% or more.

#### Field application of the subtractive method

In the last two seasons attempts have been made to carry the subtractive method a stage further, to field tests. In the pot experiments above, the major element nutrients were carefully purified to be free from any specified trace element where necessary. This cannot be done for the quantities required for field trials, and it must be assumed that trace-element impurities in the major element supplements will not be large enough to mask deficiencies. The fertilizer formula above was modified for field use, using chemicals more easily handled in the dry state. The mixtures were applied broadcast before planting, in amounts corresponding to 4 cwt. of complete mixture per acre.

The trials were carried out at four different places in Gambia, two representing moderately fertile conditions and two representing poor conditions. The complete mixture gave increased yields of groundnuts (in shell) of 50-64% over the controls (see Table III).

Omission of some of the major elements had marked effects on yield. At site 4 the omission of P, K or Ca prevented any significant increase in yield, while the complete mixture gave 587 lb. extra. At sites 2 and 3 omission of S prevented any appreciable increase, while the complete mixture gave 418 and 450 lb. extra, respectively. Lack of N at site 3 led to a reduction in yield of 400 lb. compared with that for the complete mixture.

Of the trace elements, the omission of boron gave some 200 lb. per acre less than the complete mixture. The omission of Cu had no effect at site 4, but at the other three caused a drop of about 250 lb. The omission of Zn reduced the increase by about 100 lb. at sites 1, 2 and 3, and by nearly 300 lb. at site 4. Omission of Mo had no effect on two sites, but reduced yields by 100 lb. or more at two others.



Table III

*Yield increments (lb. of dry nuts in shell per acre) compared with controls for groundnuts at four sites in Gambia*

Treatment	Site 1	Site 2	Site 3	Site 4
Complete	641	418	450	587
Omit N	500	445	52	197
Omit P	390	250	85	12
Omit K	151	40	385	2
Omit Ca	486	293	— 227	— 161
Omit Mg	473	152	318	547
Omit S	238	— 43	22	310
Omit B	458	249	203	235
Omit Cu	402	138	200	715
Omit Zn	540	293	305	290
Omit Mo	753	321	443	493

The control yields were 1020, 685, 905 and 913 lb./acre for sites 1-4, respectively

### Discussion

In terms of groundnut yields it is apparent that the omission of one or more of the trace elements might lead to a reduction of response of 200 lb. per acre or more from an otherwise suitable fertilizer mixture.

The critical significance of the results of the subtractive trials will be readily apparent. In the areas where the trials took place there existed several mineral deficiencies in the soil, each capable of causing a more or less severe restriction in yield. If the customary factorial design had been used to diagnose the deficiencies it would have been unreasonable to expect increases of more than a few per cent for any single fertilizer chemical applied alone. First-order interactions might have indicated additional deficiencies, but the combinations would have to be pursued to the 4th or 5th order to get evidence of some of the less severe deficiencies. The number of combinations involved is too great to be practicable. It is easy to see why the Gambia Department of Agriculture failed to get responses in its fertilizer trials in 1924-28. The experiments were too simple and involved too few elements.

It follows from the above results that testing for deficiencies by the application of single chemicals is not a hopeful procedure and might easily fail to show any trace-element effect at all, even though these deficiencies might be restricting responses to a fraction of their potentiality. The subtractive method minimizes the effect of possible multiple deficiencies and indicates probable specific deficiencies using as few mixtures as possible, one for each element together with a complete nutrient and a control.

It follows too that having located the deficiencies the best responses from fertilizer supplements can be achieved only by simultaneous correction of all the deficiencies. This may call for the adoption or development of a new experimental technique capable of dealing with multiple deficiencies more easily than does the usual factorial design. A possible method of dealing with all six major elements in an experiment of simple design has been proposed by Homès.<sup>6</sup> The remaining trace elements could readily be dealt with by the subtractive method. Provided care is taken not to apply trace elements in excessive, toxic amounts, no great expense is incurred in making a 'blanket' dressing to include them all, until such time as it is possible to decide which can be safely omitted from a formulation.

### Conclusions

The evidence of the widespread incidence of trace-element deficiencies in tropical areas, taken in conjunction with the indications of the magnitude of their possible effect on crop yields, serves to emphasize that the approach to the problem of fertilizer trials, at least in the tropics, needs broadening. The need for maximum efficiency is so great that it is not wise to ignore possible effects of any single essential element. The fact that the trace elements are sometimes called minor elements should not be construed to imply that their influence on plant growth and yield is minor, or that they can be overlooked temporarily while other elements are dealt with. To plants they are just as important as N, P and K. Any trial which does

not take into account the possible effect of *all* the essential elements must be regarded as incomplete, open to misinterpretation, and perhaps unrepresentative of the true potentialities inherent in the situation.

### Summary

(1) The 'Shifting System' of agriculture in the tropics is described and its requirements and limitations pointed out.

(2) The system must ultimately fail to support its dependent population. Before the failure becomes widespread a more intensive alternative must be found, capable of sustained yields.

(3) In many tropical areas crop yields are low and are not raised to maximal values by conventional fertilizers.

(4) Evidence is presented of the presence of multiple mineral deficiencies, involving trace elements as well as major elements.

(5) The need for new techniques to assess and remedy multiple deficiencies in fertilizer trials is pointed out.

(6) The outlook on fertilizer work in the tropics should be broadened to include all essential elements on an equal footing, avoiding too much emphasis on 3 or 4 major elements to the exclusion of the rest.

### References

- <sup>1</sup> Engledow, F. L., 'Report on Agriculture, Fisheries, Forestry and Veterinary Matters', West Indian Royal Commission, 1945, Cmd. 6608 (London: H.M.S.O.)
- <sup>2</sup> Ferrand, M., Bachy, A., & Ollagnier, M., *C.R. Acad. Sci. Paris*, 1951, **233**, 1218
- <sup>3</sup> Joshi, N. V., & Joshi, S. G., *Sci. & Cult.*, 1952, **18**, 9607
- <sup>4</sup> Schutte, K., 'A Survey of Plant Minor Element Deficiencies in Africa', *Afr. Soils*, 1954, III, (2), p. 284
- <sup>5</sup> Hewitt, E. J., 'Sand and Water Culture Methods used in the Study of Plant Nutrition', *Commonw. agric. Bur., E. Malling*, 1952, Tech. Commun., 22
- <sup>6</sup> Homès, M., 'L'Alimentation Minérale des Plantes et le Problème des Engrais Chimiques', 1953 (Liège: Editions 'Sciences et Lettres')

### Discussion

*Mr. J. P. Evenson* (Wellcome Research Laboratories): Full advantage of fertilizers cannot be gained unless accompanied by sound agronomic practices. In Uganda the E.C.G.C. had obtained good increases in cotton yield by taking full advantage of the available rainfall: this was without additional fertilizers. Some soils in Uganda had maintained quite reasonable yields for twenty years.

*Dr. Webb*: Not all soils are infertile and it is certainly true that good farming is essential to make full use of fertilizers; one could expect doubling and trebling of yields once the right conditions were found.

*Mr. F. C. Cooke* ('World Crops'): There is a tremendous field for trace element research in the tropics. Concentration on the major elements over the past 25 years has not resulted in very conclusive results, and this has led to the initiation of multi-nutrient research in Ceylon. The essential difference between temperate and tropical research lies in soil temperature, severe fluctuations in rainfall and the capping of the soils by heavy rain, which would affect nutrient uptake.

*Prof. E. G. Hallsworth* (Nottingham): I have noted in Australia that germination of small seeds sown in cleared soil was hindered by high temperatures which reached 127° at 1 in. depth. When temperatures rise to these levels, conditions are very unlike those found in temperate regions. Chemists need to remember the effect of high temperatures on soil processes.

*Dr. J. H. Becking* (Wageningen, Holland): I have tested tropical soils for molybdenum by means of *Aspergillus niger*. Red tropical soils contain very little molybdenum (0.05%), whereas the black soils have more (0.20%). Lateritic soils contain only one-hundredth of the citric-soluble iron present in the black soils. This work was undertaken in the study of the *Bjerrinkia* organism, which is able to fix atmospheric nitrogen in acid soils containing only one-tenth of the amount of molybdenum required by *Azotobacter*.

Dr. Webb : Some acid soils in the Gambia are still lower in molybdenum content, but nevertheless they do not always respond to addition of molybdenum.

*Dr. H. L. Richardson* (Imperial Chemical Industries Ltd.) : Dr. Webb is to be congratulated on the results he has obtained, knowing the difficulties under which he was working in the Gambia. I urge that people studying these problems in the tropics should do as many field experiments as possible, as widely dispersed as possible. Pot experiments give valuable indications, but results are tantalizing : before the pot results can be applied in practice, field experiments still have to be done. I support Mr. Evenson regarding the need to get agronomic conditions right. In multifactorial trace-element field experiments carried out in Malaya, at the cost of a good deal of trouble, disappointing responses were obtained to either major or minor elements. This was probably because of agronomic factors, including the use of paddy rice varieties of limited yielding capacity.

*Dr. E. C. Owen* : Is the value of allowing reversion to forest due to the tap roots during the dry season bringing up trace elements from deeper levels and depositing them as leaves on the surface ?

Dr. Webb : Yes.

## THE APPLICATION OF CHROMATOGRAPHY TO NUTRITIONAL PROBLEMS IN PLANTS\*

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### Introduction

Given light, air and water, the plant requires in addition to the major nutrients certain other elements, the trace elements or micronutrients. Some of these are known to be components of enzyme systems and can participate in electron-transfer reactions by virtue of their alternative valency states.

A full understanding of their function will require a much greater knowledge of the organic constituents of plants than we at present possess. Only storage and structural materials, end products of metabolism, or those whose rate of reaction is slow in comparison with their rate of formation will accumulate and be easily detected. Important intermediates and reactive substances will be present only in very small amounts since the reactions by which they are utilized are rapid and their equilibrium concentrations consequently low. In the study of such transient intermediates we may be helped by their accumulation under abnormal conditions such as nutritional deficiency: the nutritional status of plants may be reflected in the accumulation or disappearance of some metabolic intermediate.

It therefore seemed profitable that the investigation of the organic compounds of plants should be linked with the study of the effects of variations in nutritional status. Visually, the response of a plant to nutritional deficiency is variation of growth habit, general stunting, changes in leaf pigmentation such as chlorosis, and premature senescence perhaps leading to early death. Some of the changes in the organic constituents which can be inferred from these symptoms are reduction in the formation of structural materials, proteins, carbohydrates, and of chlorophyll, derangement of metabolism leading to tissue breakdown and the premature appearance of products such as anthocyanins usually associated with senescence. These visible signs are consequent upon more fundamental changes, and the purpose of this paper is to describe some experiments made with the object both of extending knowledge of plant constituents and of finding whether particular changes in the nature or amounts of various substances could be linked with specific micronutrient deficiencies.

The first set of experiments relates to variations in the free amino-acid content of the leaf of cauliflower grown under conditions of molybdenum deficiency with various forms of nitrogen supply. The variations induced by change of nitrogen source emphasized the need for some knowledge of the effect of changes in the supply of major nutrients, and so the second set of experiments consists of a more general survey of the simpler organic constituents of tomato leaf and their response to variations in nutritional status with regard to the major nutrients.

### Experimental

Cauliflower plants were grown in sand culture by methods that have been described elsewhere.<sup>1</sup> In the earliest experiments nitrogen was given as nitrate: in later experiments equivalent amounts of nitrite, ammonium salts, urea or glutamic acid were used as alternative sources. The molybdenum levels ranged from 0.00005 to 50 p.p.m. (normal level is about 0.02 p.p.m.).

The free amino-acids were extracted from leaf samples in 70% alcohol. Extracts were concentrated *in vacuo* to a degree sufficient to permit the use of amounts between 5 and 20  $\mu$ l. for paper chromatography. Chromatograms were run on Whatman No. 1 paper by the descending method, using either butanol-acetic acid or phenol-ammonia solvents for single-dimensional separations, and the same solvents successively in that order for two-dimensional separations. Chromatograms were dried at room temperature and developed with ninhydrin. Attempts at

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quantitative determinations of individual amino-acids from chromatograms were unpromising, so visual estimates of the relative amounts were made either within a series or by comparison with standard spots prepared at the same time as the unknowns.

Tomato plants for the second set of experiments were grown in sand culture also—controls with complete nutrient, others at deficiency levels of nitrogen, phosphorus, potassium, calcium and magnesium.

In order to examine free amino-acids, carboxylic acids and simple carbohydrates, the leaf extract (made this time in 25% alcohol) was separated into three fractions by successive passages through cation-exchange resin (Zeokarb-225) which removes amino-acids, and anion-exchange resin (Amberlite-IRA-400, carbonate form) to remove the carboxylic acids; carbohydrates pass through both columns. The amino-acids were eluted with ammonia and the carboxylic acids with ammonium carbonate solution; the latter was passed again through cation-exchange resin. The solutions containing the various fractions were concentrated at room temperature for chromatographic examination.

The method of chromatography of the amino-acids has already been described. A comparison between direct 70% alcoholic leaf extracts and corresponding Zeokarb eluates showed no difference in amino-acid pattern. Visual assessments of spot strengths were made as before. Carboxylic acids were examined by paper chromatography using both benzyl alcohol and ethanol-ammonia solvents, with a bromocresol green spray to reveal the spots. The acids present were estimated by the spot-weight method described by Phillips *et al.*<sup>2</sup>

For carbohydrates, the most generally useful solvent was found to be *n*-butanol saturated with water at room temperature and used at 50°. Butanol-acetic acid and ethyl acetate-pyridine, used at room temperature, were used for confirmatory work. Trevelyan's silver reagent<sup>3</sup> and *p*-anisidine hydrochloride<sup>4</sup> were both used for development of the spots.

Only visual estimates of the relative intensities of spots have been made.

## Results

### *Cauliflower*

In the first experiments, nitrate alone was used as the nitrogen source. Glutamic acid content was consistently depressed by molybdenum deficiency, while alanine, aspartic acid and glutamine showed similar but less pronounced changes. Other amino-acids observed included asparagine, valine, serine, threonine and the leucines: they were present in much lower concentrations and showed less variation but usually in the same direction.

Cysteic acid, or a labile precursor, was found in the mature leaves of all plants but in the young leaves of molybdenum-deficient plants only.

With nitrogen sources other than nitrate the content of glutamic acid at low molybdenum levels was found to be reduced in one season's plants but not in those of the following season. The most striking and consistent effect was on arginine, which is a minor constituent in 'nitrate' plants. Plants grown with reduced nitrogen sources—ammonia, urea, glutamic acid—showed an enhanced arginine content at normal levels of molybdenum, and at low level of molybdenum it showed a further large increase.

Serine, also a minor constituent of 'nitrate' plants, shows similar but less pronounced changes.

### *Tomato*

*Amino-acids.*—The basic pattern of free amino-acids found in tomato leaf includes all those found in cauliflower, with the exception of arginine; in addition proline and  $\gamma$ -aminobutyric acid are commonly present.

Nitrogen deficiency, as might be expected, lowers the whole amino-acid content. From an extract prepared under the same conditions as those of the control, only much weakened spots of glutamic and  $\gamma$ -aminobutyric acids can be seen on a chromatogram; all the other amino-acids are reduced below the threshold of detection.

The amino-acid patterns found with various nutrient deficiencies are set out in Table I.

Table I

*Amino-acid patterns in tomato leaves with nutrient deficiencies*

	Control	Deficiency of				
		N	P	K	Ca	Mg
Aspartic acid	++		++	++	++	++
Glutamic acid	++	+	++	++	++	++
Serine	++			++	++	++
Asparagine	++		++	+++	+++	+++
Threonine	++			++	++	++
Glutamine	++		++	+++	+++	+++
Alanine	++		++	+++	+++	+++
Proline	++		++	++	++	+++
$\gamma$ -Aminobutyric acid	++	+	++	++	++	++
Valine	++		++	++	++	++
Leucines	++		++	++	++	++

+ Weak spot    ++ Normal spot    +++ Strong spot    Blank space = absent  
Control spot assessed as normal for each substance

*Carboxylic acids.*—Only malic and citric acids have been found. The chromatograms show also some streaky spots of low  $R_F$  which appear to be due to phosphate. Average contents of these acids found in leaves from the various treatments are given in Table II.

Table II

*Acids (mg./100 g.) in tomato leaves with nutrient deficiencies*

		Deficiency of				
		N	P	K	Ca	Mg
Malic acid	534	125	394	161	562	430
Citric acid	560	97	858	174	446	88

*Carbohydrates.*—Fructose, glucose, sucrose, inositol and galactose have been observed in all extracts examined. From visual assessment of the spot strengths, there appears to be a large increase in the content of carbohydrates in the calcium- and magnesium-deficient samples; but so far as can be judged in the absence of quantitative results the effect is more a general increase in all the substances rather than an accumulation of particular individuals involving a change in their relative proportions.

## Discussion

The most clearly marked and consistent nutritional effect, and the first one observed in molybdenum-deficient cauliflower leaf with nitrate as nitrogen source, is the depression of glutamic acid. As nitrate accumulates in cauliflower under these conditions and as molybdenum is known to be a component of the nitrate reductase system in *Neurospora*, a lowering of amino-acid content is to be expected; glutamic acid, which occupies a central position in schemes of amino-acid metabolism, seems to be most consistently reduced in amount.

If nitrogen is fed in other forms, the nitrate reductase step can no longer restrict the supply in molybdenum-deficient plants. However, changes in amino-acid pattern are still observed: the consistency of the glutamic acid decrease is lost although the amounts still vary, and a consistent increase in arginine is found, accentuated by molybdenum deficiency. The increase in arginine content, though enhanced by molybdenum deficiency, cannot be solely attributed to this cause. The changes in serine, and lesser changes in other amino-acids, emphasize the complexity of the factors involved in the balance of amino-acids.

The effects of major-element deficiencies on the tomato leaf amino-acids show that dissimilar causes can lead to rather similar effects. Thus deficiencies of potassium, magnesium and calcium all lead to increases in the amounts of asparagine and glutamine, although differing in their effects on other amino-acids. It is perhaps significant that these deficiencies, with a normal nitrogen supply, increase the production of amides which are a form of nitrogen storage, whereas in the cauliflower, molybdenum deficiency with non-nitrate nitrogen increases the formation of arginine, another compound of very high nitrogen content and perhaps a means of storage.

Possibly the initial specific effect of various deficiencies may lead to widespread dislocations of linked metabolic processes, whose ultimate manifestations are much more similar than the first causes.

The conflicting results concerning the effect of molybdenum deficiency on glutamic acid in non-nitrate plants in two successive seasons is a reminder that variations in environment as well as in nutrition must be taken into account.

Little comment can be made on the results of the examination of the carboxylic acid and carbohydrate fractions from tomato leaf, other than to point to the contrast between the apparent variation in both absolute and relative amounts of the acids and the indications of little change in relative proportions of the carbohydrates.

### Conclusions

The results so far obtained do not lend much support to the idea that specific changes in the overall pattern of organic constituents in deficient leaf tissue are likely to be found corresponding to various mineral deficiencies. They do, however, emphasize the complexity of interlinkage of the various processes within the plant. The response of these processes to deficiencies will need to be studied in isolation and in a controlled environment.

### Acknowledgment

Thanks are due to Dr. E. J. Hewitt for the provision of the plant material grown under various conditions of nutritional deficiency.

### References

- <sup>1</sup> Agarwala, S. C., & Hewitt, E. J., *J. hort. Sci.*, 1954, **29**, 278; 1955, **30**, 163; Hewitt, E. J., *Bur. Hort. E. Malting*, 1952, Tech. Commun. 22
- <sup>2</sup> Phillips, J. D., Pollard, A., & Whiting, G. C., *J. Sci. Fd Agric.*, 1956, **7**, 31
- <sup>3</sup> Trevelyan, W. E., Procter, D. P., & Harrison, J. S., *Nature, Lond.*, 1950, **166**, 444
- <sup>4</sup> Hough, L., Jones, J. K. N., & Wadman, W. H., *J. chem. Soc.*, 1950, p. 1702

### Discussion

*Dr. Hewitt*: Arginine accumulates in molybdenum-deficient plants when the nitrogen source is other than nitrate. Can Mr. Williams comment on this in view of the high nitrogen content both of arginine and of amides that also accumulate under certain deficiency conditions.

*Mr. Williams*: I cannot add to what is said in my paper; arginine under conditions of molybdenum deficiency and non-nitrate-nitrogen supply may perhaps be a form of nitrogen storage alternative to the amides whose accumulation is typical of certain other nutritional states.

*Dr. Nicholas*: It is interesting to note that molybdenum deficiency affects catalase and peroxidase indirectly, because the deficiency reduces the activity of some flavin enzymes that produce their common substrate, hydrogen peroxide.

*Dr. Ducet*: Using radiocarbon-14 I have found that the sugar picture is changed within a few minutes under conditions of molybdenum deficiency, polysaccharides being formed very quickly; amino-acids are also disturbed.

*Dr. D. P. Cuthbertson* (Rowett Institute): Animal nutritionists must be very interested in the findings of Mr. Williams. They would naturally wish to know the proportion of the free amino-acids in relation to the total free and bound amino-acids present in the tissues of the normal and deficient plants. For non-ruminants, in particular, information on the changes in essential amino-acids would be of interest. In animal tissues the accumulation of free amino-acids consists largely of the unessential amino-acids, presumably in process of metabolic change to other compounds.

*Mr. Williams*: I was looking at differences between amino-acids the plants can use and those they cannot, or rather the balance between protein build-up and breakdown processes. I have presented a simple picture of a very complex situation. In reply to a remark by *Professor Wallace*, he added that he was indeed much interested in polyphenols but had not as yet any information in relation to their nutritional effects or variations.

**General discussion**

*Dr. J. M. S. Reith* (Macaulay Institute) : Has Dr. Nicholas or any other of the Long Ashton workers any information as to whether manganese and copper are partly interchangeable in the nutrition of plants? We have found in some of our experiments with oats a response to manganese in the absence but not in the presence of copper. *Dr. Hewitt* : No.

*Dr. E. C. Owen* : What is the relation between physiological availability of metal chelates and their dissociation constants? Since neither bacteria nor yeasts could use iron in chelated forms, would a high stability constant make the iron in a chelate unavailable to the plant?

*Dr. Bould* : Although the ferric chelates are very stable, they are reduced to ferrous iron by the action of light; the ferrous chelates so formed are less stable than those of some other metals and their iron can be liberated.

*Dr. Mitchell* (Macaulay Institute) : Whereas ionic gallium and indium cause chlorosis, the presence of considerable amounts of these elements in chelated form does not. It appears therefore that these are not decomposed by the action of light.

*Dr. Nicholas* : Sodium-EDTA can produce a trace-metal deficiency in micro-organisms grown in culture solutions containing the chelate because the metal-EDTA complexes are not readily absorbed.

*Dr. Bould* : A similar effect is produced in higher plants by sodium-EDTA whereas no such effects are produced by the iron chelates.



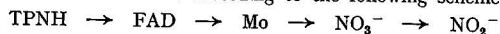
## THE USE OF PAPER CHROMATOGRAPHY IN THE SEPARATION OF THE VALENCY STATES OF BIOLOGICALLY IMPORTANT METALS\*

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The separation of a number of metal-valency states by paper chromatography was a necessary preliminary to an investigation of the rôle of these valencies in the metabolism of micro-organisms. Separations were obtained between: Mo<sup>VI</sup> and Mo<sup>V</sup>, Mo<sup>V</sup> and Mo<sup>III</sup>, V<sup>V</sup> and V<sup>IV</sup>, V<sup>IV</sup> and V<sup>III</sup>, Co<sup>III</sup> and Co<sup>II</sup>, Fe<sup>III</sup> and Fe<sup>II</sup>, Mn<sup>VII</sup>(MnO<sub>4</sub><sup>-</sup>) and Mn<sup>III</sup>, Mn<sup>III</sup> and Mn<sup>II</sup>, Cu<sup>II</sup> and Cu<sup>I</sup>. Those between Mo<sup>VI</sup> and Mo<sup>V</sup> as thiocyanates, and Fe<sup>III</sup> and Fe<sup>II</sup> as acetates, appeared to be quantitative. The preparation of 8-hydroxyquinoline complexes of Mo<sup>V</sup>, Fe<sup>II</sup> and Cu<sup>I</sup> is also described.

The rôle of trace metals in cell metabolism has been investigated in recent years, especially of those whose valency states function in electron transfer in biochemical systems. Thus metals which are essential constituents of enzyme systems in certain instances undergo valency changes during enzyme action. Nicholas & Nason<sup>1</sup> found that Mo is a constituent of nitrate reductase and mediates the transfer of electrons according to the following scheme:



where TPNH = reduced triphosphopyridine nucleotide

FAD = flavin adenine dinucleotide.

The change in Mo valency in the above sequence has been shown to involve Mo<sup>VI</sup> and Mo<sup>V</sup>.<sup>2</sup>

One of the rôles of Fe in metabolism is associated with the cytochromes which contain both ferrous and ferric iron. The results of recent experiments indicate that Fe is also an essential constituent of hydrogenase in *Azotobacter* and *Clostridium*.<sup>3</sup>

Tyrosinase<sup>4</sup> and ascorbic acid oxidase<sup>5</sup> both contain Cu and catalyse respectively the oxidation of catechol → halochrome pigments → melanin, and ascorbic acid → dehydroascorbic acid. During these oxidations a change in valency of the Cu was postulated. Butyryl co-enzyme A dehydrogenase<sup>6</sup> which functions in fatty acid metabolism, and laccase<sup>7</sup> which is present in *Rhus* leaves, are also Cu-containing enzymes. The results of recent experiments show that Cu deficiency markedly reduces nitrite reductase in *Neurospora*.<sup>3</sup>

Mn is an essential trace element and can substitute for Mg in the enzymic breakdown of sugars in plant and animal tissues. It has been shown that Mn undergoes a number of valency changes in soil.<sup>8,9</sup>

The work described in this paper was undertaken with a view to determining the rôle of valency changes in metals during metabolism. Pure solutions of metals in various valency states were supplied to micro-organisms grown in pure culture. Certain properties of the systems such as enzyme activity, the amount of metal taken up by the organism from a given valency solution, etc., were then determined.

Analysis by paper chromatography has become widely used in the field of inorganic chemistry over the past few years, and the separations which have been achieved between cations have been numerous. The separation of different valency states of a single metal is a recent development.

Pollard and co-workers have reported good separations between the valency states of U and Fe<sup>10</sup> using eluants consisting of ether, methanol, hydrochloric acid and water, and further exploratory experiments<sup>11</sup> yielded separations between Cu<sup>I</sup> and Cu<sup>II</sup>, As<sup>III</sup> and As<sup>V</sup>, Sb<sup>III</sup> and Sb<sup>V</sup>, Cr<sup>III</sup> and Cr<sup>VI</sup>, Mo<sup>V</sup> and Mo<sup>VI</sup>. The same workers also reported that similar solvent systems would separate Hg<sup>I</sup> and Hg<sup>II</sup>, Pt<sup>II</sup> and Pt<sup>IV</sup>, and V<sup>IV</sup> and V<sup>V</sup> but no details were given.

For the preparation of solutions to be used in biological experiments in these laboratories analysis by paper chromatography was essential and an account of this work is given below. The metals examined include Mo, V, Co, Fe, Mn and Cu and the separations of the valencies

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were effected by downward chromatography upon acid-washed Whatman No. 1 filter paper strips. Eluants for the separations were prepared by mixing a 'lipophilic' liquid (e.g., an alcohol or ketone) with a 'hydrophilic' one (e.g., water) in varying proportions, and noting the composition that gave the best separation. The 'lipophilic' nature of the mixture could then be increased by replacing the alcohol or ketone by a higher homologue or (to a greater extent) by an ether. Similarly the 'hydrophilic' properties could be enhanced by the addition of acids of varying strengths or salts. In this way, by methodically altering these two opposing characteristics, an eluant which gave the best resolution for the system used was obtained.

**Molybdenum.**—Mixtures of hexa- and penta-valent Mo (in the presence of sufficient mineral acid to prevent the formation of molybdenum blue) were separated as (a) thiocyanates,<sup>12</sup> and (b) as 8-hydroxyquinoline (oxine) complexes.<sup>13</sup> In (a) an eluant of water, acetic acid, and potassium thiocyanate was used; the spots were developed with either a mixed stannous chloride-potassium thiocyanate reagent (orange spots; minimum detected: 2  $\mu\text{g}$ . of either valency<sup>14</sup>) or dithiol and hydrochloric acid (green spots; minimum detected: 0.5  $\mu\text{g}$ . of either valency<sup>14</sup>). The  $R_F$  values were 0.3–0.4 for  $\text{Mo}^{\text{VI}}$  and 0.8–0.9 for  $\text{Mo}^{\text{V}}$  and, using the pilot-strip technique of Pollard *et al.*,<sup>15</sup> the separation was found to be quantitative within an error of approximately  $\pm 1.5\%$  for amounts of 50–60  $\mu\text{g}$ . of each valency state.<sup>12</sup> Mo in solutions and fractions was estimated by heating them with potassium thiocyanate and hydrochloric acid and extracting the resulting  $\text{Mo}^{\text{V}}$  thiocyanate into a known volume of ethyl acetate. The colour intensity of the extract was measured at 490  $m\mu$  on a Hilger Spekker, calibrated over the concentration range 1–14  $\mu\text{g}$ . Mo per ml.

In (b)  $\text{Mo}^{\text{VI}}$  and  $\text{Mo}^{\text{V}}$  oxinates were prepared by adding ethanolic oxine to solutions of sodium molybdate (acidified by hydrochloric acid) and molybdenum pentachloride in 10N-hydrochloric acid respectively.<sup>13</sup> The  $\text{Mo}^{\text{V}}$  complex was precipitated from the mixture by the addition of water, and the complexes were separated by filtration and washed with water and ether. A mixture of the oxinates (in chloroform solution) was resolved using an eluant of ethanol. The  $\text{Mo}^{\text{VI}}$  compound (a yellow solid with absorption maxima at 250, 370  $m\mu$  and a minimum at 280  $m\mu$ ) had an  $R_F$  value of 0, and the  $\text{Mo}^{\text{V}}$  compound (a dark purple solid with absorption maxima at 243, 540  $m\mu$  and a minimum at 480  $m\mu$ ) an  $R_F$  value of 0.9. The resolution was due possibly to solvent extraction of the  $\text{Mo}^{\text{V}}$  compound by ethanol rather than by true chromatography, as the  $\text{Mo}^{\text{VI}}$  complex is very sparingly soluble in this solvent whereas the  $\text{Mo}^{\text{V}}$  compound dissolves to a certain extent. Although the separation was not quantitative (recovery of  $\text{Mo}^{\text{V}}$  was on average 7% below the theoretical value), the method provided a quick and efficient qualitative test for Mo valency-purity in a solution. For this the latter was acidified (HCl) and treated with an excess of ethanolic oxine. The mixture was shaken with chloroform and water and allowed to stand. A purple colour in the chloroform layer indicated the presence of  $\text{Mo}^{\text{V}}$ , and  $\text{Mo}^{\text{VI}}$  was detected by a short paper-strip run in ethanol as described above.

Penta- and tri-valent Mo were separated using an eluant of thiocyanate in ether.<sup>13</sup>  $R_F$  values were 0.8 for  $\text{Mo}^{\text{V}}$  (and Zn, see below) and 0 for  $\text{Mo}^{\text{III}}$ . A solution of  $\text{Mo}^{3+}$  was prepared by reducing an acidified solution of sodium molybdate with zinc dust and afterwards removing traces of  $\text{Mo}^{6+}$  and  $\text{Zn}^{2+}$  ions by elution from a cellulose column with the thiocyanate-ether solvent. The pure  $\text{Mo}^{3+}$  was recovered from the column by washing the latter free of thiocyanate with ether, followed by elution of the Mo fraction with distilled water. The solution of  $\text{Mo}^{\text{III}}$  could not be kept longer than 24 hours in a bottle stored in the refrigerator, but could be preserved in an atmosphere of  $\text{CO}_2$  in a sealed tube. The valency state of the Mo in the zinc-reduced solution was checked by reducing a known amount of molybdate and titrating the solution with standard potassium permanganate.<sup>13</sup>

The lack of movement of  $\text{Mo}^{3+}$  in the eluant may be due to the adsorption of this relatively ionic form of the metal by the cellulose while the more covalent  $\text{Mo}^{\text{V}}$  thiocyanate (and zinc thiocyanate) dissolve in, and move with, the organic eluant. A separation between  $\text{Mo}^{6+}$  and  $\text{Mo}^{3+}$  is not feasible as ions with these valencies interact giving  $\text{Mo}^{6+}$ .

The above separations were applied to the problem of determining the lowest Mo valency present in a dithionite-reduced solution of sodium molybdate. Using a 1:10 molar ratio of  $\text{MoO}_4^{2-}$ :  $\text{S}_2\text{O}_4^{2-}$  it was shown by resolution of the oxinates that a mixture of  $\text{Mo}^{\text{V}}$  and  $\text{Mo}^{\text{VI}}$  (the presence of which precludes  $\text{Mo}^{\text{III}}$ ) was produced. When the molar ratio of  $\text{MoO}_4^{2-}$ :  $\text{S}_2\text{O}_4^{2-}$

was 1 : 20, a resolution of the thiocyanates showed that some  $\text{Mo}^{\text{VI}}$  was present but  $\text{Mo}^{\text{V}}$  predominated. At a ratio of 1 : 40, however,  $\text{Mo}^{\text{VI}}$  was not detected. Chromatographic analysis of this mixture in the thiocyanic acid-ether eluant showed that  $\text{Mo}^{\text{III}}$  was absent. The lowest valency in the system therefore is  $\text{Mo}^{\text{V}}$ .

*Vanadium.*—Vanadium in different valency states was separated using eluants consisting of ethanol or acetone with acetic acid, water and sodium acetate.<sup>16</sup> Variations in the quantities of the constituents were required in order to produce good separations between  $\text{V}^{\text{V}}$  and  $\text{V}^{\text{IV}}$ , and between  $\text{V}^{\text{IV}}$  and  $\text{V}^{\text{III}}$ . The pH of the latter mixture had to be adjusted to 6–7 to produce a satisfactory resolution. A separation between  $\text{V}^{5+}$  and  $\text{V}^{3+}$  was not possible because these ions interacted giving  $\text{V}^{4+}$ ; solutions of  $\text{V}^{2+}$  (which reacts with  $\text{V}^{4+}$  to give  $\text{V}^{3+}$ ) were too unstable to permit their use in experiments of any duration. The chromatograms were developed with an ethereal solution of oxine and  $R_F$  values were approximately 0.4–0.5 for  $\text{V}^{\text{V}}$  (black spot, slowly fading), 0.7–0.8 for  $\text{V}^{\text{IV}}$  (yellowish-brown spot, turning black) and 0.9–1.0 for  $\text{V}^{\text{III}}$  (rust-coloured spot, turning black). The rust-coloured spot was due presumably to the formation of an oxine complex of  $\text{V}^{\text{III}}$ ; by treating a solution of vanadium trichloride with ethanolic oxine a brown compound was prepared, of which the V and N content corresponded to the composition  $\text{V}(\text{C}_9\text{H}_6\text{ON})_3$ . Bielig & Bayer<sup>17</sup> have prepared and studied the oxinates of vanadium. The complete analysis of their  $\text{V}^{\text{III}}$  complex corresponded very closely to this formula.

Pure solutions of  $\text{V}^{\text{III}}$  were obtained by the reduction of acidified  $\text{VOCl}_2$  or  $\text{VOSO}_4$  solution with zinc dust followed by the removal of  $\text{Zn}^{2+}$  ions (which are toxic to micro-organisms) by elution from a cellulose column with a solvent system containing ethanol, ether and hydrochloric acid.<sup>16</sup>  $R_F$  values were  $\text{V}^{\text{III}}$ , 0.24, and  $\text{Zn}^{2+}$ , 0.94. A solution of  $\text{V}^{\text{III}}$  was also prepared by heating V metal powder in a stream of Br vapour carried by  $\text{CO}_2$ , and dissolving the black sublimate of  $\text{VBr}_3$  in a mixture of acetone and water.

Solutions of  $\text{V}^{\text{III}}$  are more stable than those of  $\text{Mo}^{\text{III}}$  and can be kept almost indefinitely in a stoppered bottle stored in a refrigerator.

A quantitative investigation was made on the  $\text{V}^{\text{V}}$ – $\text{V}^{\text{IV}}$  separation by the usual technique.<sup>15</sup> The bands bearing the vanadium on the main chromatogram were determined by wet-ashing them with nitric and perchloric acid, followed by extracting the vanadium, as the  $\text{V}^{\text{V}}$  oxine complex, into chloroform. The extracts were measured colorimetrically at 550  $\mu\mu$  on a Hilger Spekker which had been calibrated over the concentration range 1–14  $\mu\text{g.}$  of V per ml. The recovery of vanadium from the  $\text{V}^{\text{V}}$  fraction was, on average, 5% above the calculated value, and that for the  $\text{V}^{\text{IV}}$  fraction 5% below the theoretical value using 350  $\mu\text{g.}$  of each valency state.<sup>16</sup> It was suggested that the reason for this was the adsorption of  $\text{V}^{4+}$  (vanadyl) ions on to a slight 'back tail' of vanadium pentoxide which had been precipitated from the  $\text{V}^{\text{V}}$  fraction by the buffer action of the sodium acetate in the eluant.

Solutions of  $\text{V}^{\text{III}}$  underwent oxidation too readily on the chromatograms to permit a quantitative investigation to be made.

*Cobalt.*—The form in which di- and tri-valent cobalt were separated was determined by the instability of the  $\text{Co}^{3+}$  ion. Ammine complexes were not employed, as these are unlikely to function in electron transfer as readily as an ionic species. The weakly-complexed acetates were preferred, a stable solution of the triacetate (which gives the reactions of the  $\text{Co}^{3+}$  ion) being readily obtained by treating an ice-cold solution of cobaltous acetate with hydrogen peroxide and potassium bicarbonate and acidifying the mixture with acetic acid.

For the separation, an eluant consisting of ethanol, ether, acetic acid and magnesium acetate was used,<sup>16</sup> and the chromatograms were developed with either an ethereal solution of oxine (yellow spots) or thiocyanic acid in acetone (blue spots).  $R_F$  values were approximately 0.9–0.95 for  $\text{Co}^{\text{III}}$  and 0.55–0.60 for  $\text{Co}^{\text{II}}$ . The use of the very sensitive reagent 2-nitroso-1-naphthol-4-sulphonic acid<sup>18</sup> as developer showed that there was a little back-tailing of  $\text{Co}^{\text{III}}$ , due presumably to some reduction to  $\text{Co}^{\text{II}}$  on the paper during the run. It was not surprising therefore that in a brief quantitative investigation the values for the  $\text{Co}^{\text{II}}$  fractions were always high and those for the  $\text{Co}^{\text{III}}$  fractions low.

The Co valency bands, after wet-ashing with nitric and perchloric acid, were estimated colorimetrically as the blue  $\text{Co}^{\text{II}}$  thiocyanate compound in acetone at 620  $\mu\text{g.}$  on a Hilger Spekker calibrated over the range 4–40  $\mu\text{g.}$  of Co per ml.

*Iron.*—Ferrous and ferric iron were quantitatively separated from a mixed solution of the sulphates, buffered to pH 4 with sodium acetate, by an eluant containing *n*-butanol, ethanol, acetic acid and water.<sup>19</sup> Chromatograms were developed with an ethereal solution of oxine and  $R_F$  values were approximately 0.2–0.3 for Fe<sup>II</sup> and 0.8–0.9 for Fe<sup>III</sup>. In the quantitative separation, a pilot spot was not always an accurate guide to the depth of a band on the main chromatogram. The latter was therefore developed by exposure to oxine vapour carried by steam in order to avoid the risk of contamination or loss of Fe from the paper if the chromatogram was dipped into an ethereal solution of oxine. The Fe in the valency bands was determined (after ashing with nitric and perchloric acid) by extraction as ferric thiocyanate into amyl alcohol-ether, and measuring the colour at 520  $\mu$ g. on a Spekker calibrated over the range 1–8  $\mu$ g. of Fe per ml. Recoveries from 250  $\mu$ g. of each valency were within  $\pm 2.5\%$  for Fe<sup>II</sup>, and virtually complete for Fe<sup>III</sup>.

To avoid ashing with nitric and perchloric acid, attempts were made to digest the paper bearing the valency bands with a mixture of hydrochloric acid and hydrogen peroxide, followed by extraction of ferric iron as thiocyanate from the disintegrated paper. Under these conditions about half the iron from either valency band remained in the ferrous state and could not be extracted. Control experiments showed that 30–50 mg. of glucose was liberated from an area of paper of approximately 100 cm.<sup>2</sup>. Chromatographic analysis showed that an acidified solution of ferric chloride was partly reduced to the ferrous state by boiling it with glucose.<sup>19</sup> No reduction occurred in the cold. Complete destruction of the paper by nitric-perchloric acid ashing is therefore essential for accurate determinations by this method.

The iron valency separation was applied to the problem of determining the rate of oxidation of ferrous iron under conditions similar to those found in a culture medium during aeration. Several mixtures containing 25 mg. of Fe<sup>II</sup> per 100 ml. of culture medium at a pH of 7.0–7.5 were prepared. Different samples were aerated for 30 and 60 minutes, and a control solution was kept unaerated. 0.2 ml. ( $\equiv 45 \mu$ g. of Fe) of each mixture was then run on paper sheets in the eluant used to separate Fe valencies. It was estimated that after 30 minutes' aeration one third of the ferrous iron was converted to ferric, and after 60 minutes one half of the total iron given was present in the ferric state.

When a fresh Fe<sup>II</sup> spot was developed by oxine, an initial transient red coloration was observed and the formation of an Fe<sup>II</sup>-oxine complex was suspected. A brick-red compound, the analysis of which corresponded to the composition  $\text{Fe}(\text{C}_9\text{H}_8\text{ON})_2 \cdot 2\text{H}_2\text{O}$ , was prepared by treating a solution of ferrous sulphate with ethanolic oxine in an inert atmosphere.<sup>19</sup> An absorption maximum at 400  $m\mu$  and a minimum at 350  $m\mu$  was obtained with a solution of the complex in pyridine which was about the only solvent in which it was readily soluble. When heated in an inert atmosphere, the compound decomposed without melting at 110° giving ferric oxinate.

*Manganese.*—As with Co the trivalent ion is unstable and separations were obtained between the weakly complexed di- and tri-acetates using an eluant containing acetic anhydride and acetic acid.<sup>20</sup> A mixture of Mn<sup>VII</sup> ( $\text{MnO}_4^-$  in acetic acid) and Mn<sup>III</sup> (acetate) could also be resolved by this eluant, but Mn<sup>2+</sup> and Mn<sup>7+</sup> interacted to yield Mn<sup>3+</sup>. This reaction was utilized to prepare solutions of the triacetate by treating manganous acetate with potassium permanganate in acetic acid medium.

Chromatograms were developed by a solution of 'tetrabase' in acetone (blue spots) and  $R_F$  values were approximately Mn<sup>I</sup>, 0.16, Mn<sup>III</sup>, 0.94, and Mn<sup>VII</sup>, 0.48. The Mn<sup>VII</sup> spot remained moderately stable during a short run (5–7 cm.) but tended to be reduced gradually to Mn<sup>III</sup> and Mn<sup>I</sup> giving short forward and backward 'tails', hence the  $R_F$  values quoted are for the main spot of each valency. Reduction was increased if either a stronger (formic) or a weaker (propionic) acid was used to replace acetic acid in the eluant. Ethers, ketones and alcohols could not be used to provide the 'lipophilic' portion of the eluant because they caused the higher valencies to decompose; hence acetic anhydride was chosen and found to be the most satisfactory.

*Copper.*—A good separation of Cu<sup>I</sup> and Cu<sup>II</sup> was obtained by Pollard, McOmie, & Banister<sup>11</sup> using a methanol-ether-hydrochloric acid-water eluant, but some oxidation of univalent copper took place, possibly because the polar state of the eluant tended to keep the copper in ionic form and made it susceptible to oxidation. When a less polar eluant containing ethanol, ether and acetic acid was used and the run was carried out in a carbon dioxide atmosphere, oxidation and

back-tailing were eliminated.<sup>20</sup> Copper was used as the Cu<sup>I</sup> and Cu<sup>II</sup> chlorides which were dissolved in a mixture of hydrochloric acid and acetone. The chromatograms were developed first with sodium molybdate solution to show Cu<sup>I</sup> (blue spot) and then with a solution of rubeanic acid in ether and exposed to ammonia to show both valencies (olive-green spots).  $R_F$  values were approximately 0.2–0.3 for Cu<sup>II</sup> and 0.6–0.7 for Cu<sup>I</sup>.

The method was successfully applied to the problem of determining the valency state of copper in apple juice storage deposits after copper in the form of cupric sulphate had been added to the juice initially.<sup>21, 22</sup> The deposit was centrifuged and treated with 2–3 drops of 10 N-hydrochloric acid in an atmosphere of carbon dioxide. Spots of the extract were run (in carbon dioxide) in the ethanol-ether-acetic acid eluant, and when the chromatogram had been developed it was observed that virtually all the copper present was in the cuprous state.

The existence of a cuprous-8-hydroxyquinoline complex was investigated; when cuprous chloride was treated with oxine in pyridine medium in an inert atmosphere and the mixture poured into an excess of air-free water, a bright orange compound was obtained which was moderately stable when dry.<sup>20</sup> The complex was shown to contain cuprous copper (by chromatographic analysis), oxine and pyridine, but no chloride. Complete analysis of the compound suggested the composition to be similar to  $(\text{CuC}_9\text{H}_6\text{ON}, \text{C}_9\text{H}_7\text{ON})_2 \cdot \text{C}_5\text{H}_5\text{N}$ .

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#### References

- <sup>1</sup> Nicholas, D. J. D., & Nason, A., *J. biol. Chem.*, 1954, **211**, 183
- <sup>2</sup> Nicholas, D. J. D., & Stevens, H. M., *Nature, Lond.*, 1955, **176**, 1066
- <sup>3</sup> Nicholas, D. J. D., this symposium
- <sup>4</sup> Mallette, M. F., in 'A Symposium on Copper Metabolism', eds. McElroy, W. D., & Glass, B., 1950, p. 48 (Baltimore: Johns Hopkins Press)
- <sup>5</sup> Dawson, C. R., in 'A Symposium on Copper Metabolism', eds. McElroy, W. D., & Glass, B., 1950, p. 18 (Baltimore: Johns Hopkins Press)
- <sup>6</sup> Mahler, H. R., *J. Amer. chem. Soc.*, 1953, **75**, 3288
- <sup>7</sup> Sumner, J. B., & Somers, G. F., 'Chemistry and Methods of Enzymes', 1947, p. 239 (New York: Academic Press Inc.)
- <sup>8</sup> Lees, H., & Quastel, J. H., *Chem. & Ind.*, 1944, p. 237
- <sup>9</sup> Mann, P. J. G., & Quastel, J. H., *Nature, Lond.*, 1946, **158**, 154
- <sup>10</sup> Pollard, F. H., *Brit. med. Bull.*, 1954, **10**, 187
- <sup>11</sup> Pollard, F. H., McOmie, J. F. W., & Banister, A. J., *Chem. & Ind.*, 1955, p. 1598
- <sup>12</sup> Candela, M. I., Hewitt, E. J., & Stevens, H. M., *Anal. chim. Acta*, 1956, **14**, 66.
- <sup>13</sup> Stevens, H. M., *Anal. chim. Acta*, 1956, **14**, 126
- <sup>14</sup> Nicholas, D. J. D., & Stevens, H. M., in 'Inorganic Nitrogen Metabolism', eds. McElroy, W. D., & Glass, B., 1956, p. 178 (Baltimore: Johns Hopkins Press)
- <sup>15</sup> Pollard, F. H., McOmie, J. F. W., Stevens, H. M., & Maddock, J. G., *J. chem. Soc.*, 1953, p. 1338
- <sup>16</sup> Stevens, H. M., *Anal. chim. Acta*, 1956, **15**, 51
- <sup>17</sup> Bielig, H. J., & Bayer, E., *Liebigs Ann.*, 1953, **584**, 96
- <sup>18</sup> Pollard, F. H., Nickless, G., & Banister, A. J., *Analyst*, 1956, **81**, 577
- <sup>19</sup> Stevens, H. M., *Anal. chim. Acta*, 1956, **15**, 538
- <sup>20</sup> Stevens, H. M., *Anal. chim. Acta*, in press
- <sup>21</sup> Kieser, M. E., Pollard, A., & Timberlake, C. F., *J. Sci. Fd Agric.*, 1957, **8**, 15
- <sup>22</sup> Timberlake, C. F., *J. Sci. Fd Agric.*, 1957, **8**, 159

#### Discussion

*Dr. A. H. Cornfield* (Imperial College): What is meant by saying some separations are not quantitative? Does this indicate that tailing occurred? If so, this is not shown on the slides.

*Dr. Stevens*: There was some unavoidable tailing. If the oxinates of vanadium are separated under ultra-violet light, a tail of the pentavalent oxinate can be seen.

*Prof. W. E. Garner*: Does the presence or absence of tailing depend on the valency state and on the solvent used?

*Dr. Stevens*: Yes.

*Prof. Garner*: Conditions should be found to prevent a change of valency state. What is the effect of carbon dioxide?

Dr. Stevens : Carbon dioxide is inert.

*Prof. Garner* : Some of these ions are very catalytic, and the use of hydrogen is suggested.

Dr. Stevens : Its lightness compared with air was a drawback, because of the difficulty of ensuring gas-tight seals.

*Dr. P. C. van Erkelens* (Holland) : Has Dr. Stevens had any experience with quantitative determination of the spots ?

Dr. Stevens : Yes. For the determination of iron the bands are excised and the paper bearing the iron digested in nitric and perchloric acid. The acid is evaporated and the iron estimated colorimetrically as the *iso*-thiocyanate after extraction with amyl alcohol.

*Dr. van Erkelens* : Has Dr. Stevens determined the amount of iron from the area and the maximum density of the spot on the paper ? This gives a good indication of the concentration.

Dr. Stevens : Dr. Pollard of the Chemistry Department of the University of Bristol has been working on this, separating metal from metal, but I cannot say with what metals the work was done. The separation is more difficult where valency states of a single element are concerned.

*Dr. van Erkelens* thought that this should not be too difficult.

*Mr. D. V. Crawford* (Nottingham) : Will Dr. Stevens comment on the problems involved in separating metals from associated organic matter. What confidence would he have in the accuracy of the relative amounts of two valency states of one element obtained in this way after such a separation ?

Dr. Stevens : For accurate work it would depend on the quantities of metal and of organic matter that were associated. Where valencies are concerned, once the metal is separated from an organic medium, one cannot be sure what has happened to the valencies. I have done iron determinations in culture media by using visual tests for the ferrous iron remaining after aeration of the media ; for inorganic separations the presence of much organic material (for example the 'sugar fronts') is a disadvantage.

*Prof. Garner* : Have metallic valencies been directly determined by the chromatographic method in enzyme systems ?

Dr. Stevens : No, because the content of metals is too small. The indirect method has been used, i.e., the metals are prepared in a known valency state and added back to the enzyme systems.

## TRACE-ELEMENT UPTAKE BY SWEET VERNAL IN RELATION TO MINERALOGY AND NEW ZEALAND GENETIC SOIL TYPES\*

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The value of soil classification as a basis of research investigations is illustrated by correlation of measurements of molybdenum uptake by sweet vernal with molybdenum content of topsoils, molybdenum retention, and clay mineralogy of a wide range of New Zealand soils. The pattern of molybdenum uptake by sweet vernal can best be understood in terms of the nature of mineral colloids in the soils. Soil molybdenum is fixed by inclusion in structures of crystalline hydrous oxides, particularly of iron. Processes such as podzolization which limit crystallization make molybdenum more readily available.

### Introduction

Trace elements essential for the growth of animals and plants have come principally from igneous rocks. Igneous rocks of the lithosphere formed by cooling of magma derived small amounts of hydrogen from inclusions of gases but their hydrogen content is low since at the time of formation most of the earth's hydrogen was already concentrated in the hydrosphere. When, however, igneous rocks reach the surface of the earth they are acted upon by water and form compounds of greater degree of hydration. Progressive hydration of minerals goes on continuously during soil formation.

In soils, primary minerals are present chiefly in the coarser gravel, sand, and silt fractions. Outside surfaces of these larger fragments in contact with the soil solution are exposed to attack by water, carbon dioxide, hydrogen ions and other ions, and oxidizing and reducing influences, and the primary minerals are continuously breaking down. In doing so they release plant nutrients to the soil solution and form insoluble residues. This process is known as chemical weathering and the extent to which it has occurred in any soil is its weathering stage. The secondary minerals of fine particle size, formed by chemical weathering, make up the clay fractions of the soil. Clay therefore consists largely of hydrous oxides and hydrous silicates.

Because of their nature and relatively large surface area, fine particles of the clay fraction are much more active chemically than the coarser gravel, sand, and silt. The particles of secondary minerals are usually less than  $2\mu$  in equivalent spherical diameter and exhibit many of the properties of colloids. Those particles less than  $0.5\mu$  in diameter are truly colloidal. Clay particles may thus possess colloidal charges which enable them to retain ions from the soil solution and restrict the tendency of ions to be leached from the soil. Sometimes retained ions remain available as plant nutrients, at other times they are fixed in a non-available form.

Thus the composition of the primary minerals in the parent material of a soil, the rate at which primary minerals weather, and the nature and amount of soil clay each plays an important part in determining the availability of elements to plants and through them to animals. Biological activity associated with soil formation is often an effective modifying influence.

Appreciation of these facts led in the first place to the genetic system of soil classification adopted at the N.Z. Soil Bureau.<sup>1</sup> When therefore in 1949 a systematic survey was undertaken of the nature of clays of New Zealand soils it was not surprising that characteristic differences were found between the mineral colloid assemblages of different soil types.

In 1952 a comprehensive investigation into element availability from New Zealand soils was begun. This too was based on the soil classification and within a comparatively short time interesting correlations were observed.

The present paper illustrates how this interpretative approach to soil classification has aided investigations of New Zealand soil problems and in particular its usefulness by reference to results for one of the trace elements, molybdenum.

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### The soil classification approach

The soil mantle covering New Zealand is a continuum resulting from the interplay of five soil-forming factors—parent material, topography, climate, soil life, and time—and there are as many different kinds of soil as there are significantly different combinations of the soil-forming factors. In mapping soils this continuum is divided into units named soil types. Each type is based on a modal soil profile, but, since the soil varies, the pedologist establishing the type must judge what degree of variation he will admit within his unit. In order to elucidate the general picture, differences between various soil types should be measured by examination of modal areas. This has been done in the studies of both element availability and soil colloids. Modal soils have been considered and, from these, trends in soil properties have been deduced.

The general survey of soils of the North Island<sup>2</sup> followed the classification of Taylor,<sup>1</sup> and parent material was recorded for each soil unit described. In both these surveys the units are arranged in order of profile sequence. When the assessment of soil properties follows the soil profile sequence, the significance of the results is much higher than if the same number of samples had been appraised solely on the basis of geographic location.

Where possible, studies are made on suites of soils derived from a single parent material, but at various stages of development, determined by differences in rainfall or vegetation. Such suites were used by Wright & Breen (quoted by Taylor<sup>3</sup>) who, using *Aspergillus niger* as the extracting organism, showed that the copper status of North Auckland soils followed a logical pattern in terms of profile development. Since a suite is derived from one parent material, variations inherited from parent material are distinguishable from those acquired during the process of soil formation.<sup>3</sup> Hence, trends established from one suite can, in a modified way, be expected to hold for all suites derived from similar parent material.

### Nature of the soil clays

Soil clay studies in most countries have, in recent years, tended to concentrate on the platy clay minerals—hydrous micas, illite, montmorillonite and kaolin. These constituents have been found in varying amounts in certain New Zealand soils but work at the Soil Bureau showed that, in soils of an intermediate weathering stage derived from basalt, most of the clay is made up of associations of hydrous oxides of silicon, aluminium, iron and titanium whereas soils derived from andesitic volcanic ash may contain allophane as their dominant clay constituent.<sup>4, 5</sup> As the properties of these components have been studied, their importance in low concentrations in other soil types has been recognized.

Some hydrous oxides of iron and aluminium, for instance, tend to behave as positive colloids under soil conditions and are active in fixing phosphate and molybdate.

More than 20 different mineral constituents have been identified in New Zealand soil clays. An account of the mode of formation of mineral colloids from primary silicates, the conditions under which they are formed and their relation to soil classification is given by Fieldes & Swindale.<sup>6</sup> Results obtained by various methods of study are described in a series of papers,<sup>5-8</sup> which demonstrate that the assemblage of mineral colloids in any soil is characteristic of its genetic soil type. These results are used in the subsequent discussion of molybdenum.

### Element availability

Wells<sup>9</sup> describes the methods that are being employed at the Soil Bureau for the study of element availability from New Zealand soils. Because of the importance of grasslands farming in New Zealand, element availability to grasses is being first considered. The grass sweet vernal (*Anthoxanthum odoratum* L.) was selected as a plant indicator of element availability because it is widespread and can be obtained from sites which have not been top-dressed, on both fertile and infertile soils under a wide climatic range. Sweet vernal is found on all major soils of New Zealand except extremely saline gley soils and drier brown-grey earths. It is distributed on all New Zealand main islands, between latitudes of 34½° S. and 47° S., from sea level to altitudes of 4000 ft., under rainfall ranging from 18 in. to over 100 in. per annum, and at average mean temperatures ranging from 42° F to 60° F. Samples of the grass at the flowering stage are analysed for as many elements as possible using a comprehensive scheme



of analysis described by Wells.<sup>9</sup> Spectrographic analysis of the plant ash by the method described by Farmer<sup>10</sup> is used for determination of calcium, sodium, magnesium, copper, manganese, iron, barium, strontium, aluminium, silicon, titanium and molybdenum. Spectrographic analysis of a concentrate from an oxine precipitate<sup>11</sup> is used for molybdenum, cobalt, gallium, vanadium, zinc, germanium, nickel, beryllium, chromium, tin, lead, silver, thallium and titanium. A flame photometer is used to determine potassium, and phosphorus is measured colorimetrically. Some results of this work have been published<sup>9,12</sup> for the composition of sweet vernal from 250 soil types.

In recent years it has been shown that in some districts of New Zealand small applications of molybdate result in marked growth responses in pastures and crops.<sup>13</sup> On account of this, the rôle of molybdenum in New Zealand soils has been studied and data for this element are chosen to illustrate the relationships discussed here.

**Molybdenum in New Zealand intrazonal soils**

Parent minerals are important in determining the nature of secondary minerals in soil clays<sup>6</sup> and it is appropriate to consider first intrazonal soils in which soil type is determined more by the nature of parent material than other soil-forming factors.

*Soils from volcanic ash*

The main primary minerals in ash soils are volcanic glass, feldspar and quartz. The soils increase in rate of weathering with increasing basicity. The course of weathering is determined by the nature of their primary silicates. Both glasses and feldspars are weathered first to amorphous hydrous oxides of very fine particle size.<sup>6</sup> With age, crystal growth and the formation of layer silicates by resilication may occur.<sup>5,6</sup>

(a) *Yellow-brown pumice soils* (genetic no. 13) are derived from rhyolitic pumiceous ash. Table I lists the average topsoil content of molybdenum in New Zealand soils derived from various parent materials. Values for cobalt, copper and manganese are listed for comparison, since these are also essential trace elements. Soils derived from this parent material have provided a classical case of cobalt deficiency causing bush sickness in stock. It will be seen from Table I that topsoils from rhyolitic pumice contain little cobalt compared with most other soils. Molybdenum content on the other hand is not conspicuously high or low.

**Table I**

*Total element content of topsoil of New Zealand soils*

Soil type	No. of samples	Average content of trace element (p.p.m. on oven-dry weight)			
		Mo	Co	Cu	Mn
Greywacké	12	1.5	3	30	700
Loess	13	1.5	7	13	1000
Sandstone	12	1.5	9	17	1000
Podzols	12	<1	<1	6	50
Calcareous	13	1.5	24	38	2000
Rhyolitic pumice	13	2	<1	6.5	1300
Andesitic ash	14	1.5	20	100	1600
Basalt	14	5	30	65	1300

Table II lists yellow-brown pumice soils in an age sequence and gives values for total plant and topsoil molybdenum together with figures for molybdenum retention by the topsoil (Wells<sup>12</sup>). With increasing age of the three main rhyolitic pumice showers, Kaharoa, Taupo, and Gisborne, there is increasing degree of weathering and increasing molybdate retention. Molybdenum uptake by sweet vernal tends to decrease with increasing weathering, increasing age and increasing molybdate retention. The oldest shower has the highest molybdate retention and the lowest plant molybdenum.

Because of their youth these soils are relatively little weathered. They contain 1-10% of inorganic clay which is made up predominantly of members of the allophane series. The

**Table II**

*Soils from rhyolitic pumice: age sequence of yellow-brown pumice soils, genetic class 13 (Wells<sup>12</sup>)*

Soil type	Ash shower	Age, years	Mo in topsoil, p.p.m.	Mo in plant, p.p.m.	Mo retention, %
Oropi sand	Kaharoa	800*	2	1.8	4
Taupo deep sand	Taupo	1700†	< 1	0.9	6
Mamaku sandy silt	Taupo	1700†	3	0.9	16
Gisborne sandy loam	Gisborne	> 1700	3	0.3	36

\* Fergusson & Rafter<sup>14</sup>

† Fergusson & Rafter<sup>15</sup>

mineral colloids of volcanic ash soils have been described by Birrell & Fieldes<sup>4</sup> and by Fieldes.<sup>5</sup> With increasing age of the soils, amorphous hydrous silica content of their clays decreases and allophane content increases. Allophane is strongly retentive of phosphate and molybdate and from the data of Table II it appears possible that in yellow-brown pumice soils competition for molybdate by allophane influences the level of molybdenum in the plant.

(b) *Yellow-brown loams* (genetic no. 14). Properties of yellow-brown loams from andesitic ash may be illustrated by soils from three ash showers recognized in the Egmont district. These, in order of increasing age, are Burrell, Stratford, and Egmont.<sup>12</sup> Data for these soils are given in Table III which also indicates the stage of development of the soils. Molybdenum retention increases with age and leaching. Topsoil molybdenum is not low. Plant molybdenum levels, though tending to decrease with increasing soil maturity, are not outstandingly low.

**Table III**

*Soils from andesitic ash, Egmont: age sequence of yellow-brown loams, genetic class 14 (Wells<sup>12</sup>)*

Soil type	Ash shower	Stage of development	Mo in topsoil, p.p.m.	Mo in plant, p.p.m.	Mo retention, %
Burrell gravelly sand	Burrell	young	2	0.8	2
Stratford sand	Stratford	semi-mature	1	1.7	22
Patua sandy loam		later semi-mature	3	1.0	40
Egmont black loam	Egmont	immature	2	1.1	10
New Plymouth black loam		semi-mature	2	0.8	30

Birrell & Fieldes<sup>4</sup> have shown that clay fractions of yellow-brown loams derived from andesitic ash contain high amounts of allophane which retains molybdate. These soils contain variable amounts of hydrous iron oxides<sup>5</sup> which also retain molybdate. The persistence of relatively high molybdenum in the plants shows that, as in the young basalt soils mentioned below, there has been insufficient time for crystallization processes to fix all the soil molybdenum.

(c) *Brown granular clays* (genetic no. 15). Values for brown granular clays on old andesitic ash and tuff are given in Table IV. Values for brown granular clays derived from andesite in the strong weathering zone are listed for comparison. Plant molybdenum levels from these soils are lower than those from yellow-brown loams on young andesitic ash though topsoil contents are similar. In these soils the predominant clay mineral is meta-halloysite and iron oxide content is high.

**Table IV**

*Brown granular clays, genetic class 15, from andesitic parent material (Wells<sup>12</sup>)*

Soil type	Parent material	Stage of development	Mo in topsoil, p.p.m.	Mo in plant, p.p.m.	Mo retention, %
Hamilton clay loam	andesitic ash	semi-mature	3	0.5	16
Parua clay	andesitic tuff	semi-mature	1	0.5	60
Awapuku clay loam	andesite and andesitic-basalt rock	immature	2	0.7	12
Awapuku clay		later immature	2	< 0.1	46
Waitakere clay		semi-mature	6	< 0.1	44
Awarua clay		later semi-mature	2	< 0.1	48

In brown granular clays from andesitic ash and tuff it has been shown that meta-halloysite has been formed by crystal growth from amorphous oxides of silicon and aluminium.<sup>5</sup> It is probable that during development of these soils iron oxides have also grown in crystal size and as in mature basalt soils molybdate has become partly fixed within such crystals.

*Basalt flow soils (included in genetic no. 16)*

Intrazonal basalt soils, in the strong weathering zone, have been arranged in a sequence beginning with the youngest soil, Ohaewai bouldery silt loam, and ending with a mature iron-stone soil, Okaihau gravelly friable clay.<sup>16</sup> As these soils approach maturity amorphous hydrous oxides of aluminium, titanium and iron partly crystallize to gibbsite, anatase and goethite.<sup>7</sup> It can be seen from Table V that decrease in surface area associated with crystal growth from amorphous oxides reduces molybdate retention and in this process molybdenum in the soil is made unavailable to the plant. Although young basalt soils have very high molybdate retentions the plant is able to obtain adequate molybdenum. It is therefore the form, rather than the amount, of hydrous oxides that gives retentive capacity and it is thus crystallization that limits molybdenum availability by fixation.

**Table V**

*Soils from basalt flow, genetic class 16 (Wells<sup>12</sup>)*

Soil type	Genetic no.	Degree of leaching	Mo in topsoil, p.p.m.	Mo in plant, p.p.m.	Mo retention, %
Ohaewai bouldery silt loam	16a	weak to moderate	8	3.1	50
Ohaewai silt loam	"	" "	8	1.2	60
Kiripaka bouldery silt loam	"	" "	6	0.6	28
Whakapai clay loam	"	" "	6	0.3	30
Waiotu friable clay	16b	strong	5	<0.1	30
Kerikeri friable clay	"	" "	7	<0.1	15
Tarairé gravelly friable clay	16c	strong with iron-stone nodules	6	<0.1	20
Okaihau gravelly friable clay	"	" " " "	7	<0.1	25

**Molybdenum in New Zealand zonal and azonal soils**

The rôle of molybdenum in zonal and azonal soils may be illustrated by consideration of a sequence of soils derived from the sedimentary rock greywacké (Table VI). Under conditions of weak to moderate weathering, soils have not accumulated hydrous oxide weathering products and consequently their capacity to retain molybdate is low and plant molybdenum is high. Topsoil molybdenum contents at this stage of soil development are low. Under moderate weathering, molybdate retention and plant molybdenum drop but topsoil molybdenum increases. With strong weathering molybdate retentions are high.

**Table VI**

*Soils from greywacké (Wells<sup>12</sup>)*

Soil type	Genetic no.	Classification*	Mo in topsoil, p.p.m.	Mo in plant, p.p.m.	Mo retention, %
Omarama sandy loam	1b	BGE	1	1.1	5
Haldon stony silt loam	21b	YGE	1	1.3	5
Hurunui stony loam	21c	YGE-YBE	1	2.8	5
Kaikoura loam	4	YBE weakly weathered	1	1.8	10
Korokoro silt loam hill soil	5b	YBE moderately weathered	2	0.2	9
Te Ranga clay loam	21e	YBE strongly weathered	3	<0.1	24
Marua clay loam	6b	YBE strongly weathered	3	0.1	24
Rangiora silty clay loam	6c	YBE strongly weathered, weakly podzolized	1	0.2	8
Hukerenui silt loam	7b	YBE strongly weathered, moderately podzolized	1	0.2	8

\* BGE = brown-grey earth  
 YGE = yellow-grey earth  
 YBE = yellow-brown earth

The kauri tree (*Agathis australis*) in the strong weathering zone has brought about podzolization of the greywacké soils; iron and aluminium have passed down the profile and molybdenum retention is decreased. This results in a slight rise in molybdenum level of the plant even though podzolization has decreased the amount of topsoil molybdenum.

### Conclusion

Consideration of soil properties in terms of systematic soil classification is useful in the study of trace element problems. It does not obviate the need for conventional methods of trace element investigation, such as field trials, but when combined with these it is possible to interpret the results in a logical way which benefits both soil classification and agricultural extension work.

### Acknowledgments

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The work described was carried out at Soil Bureau, Department of Scientific and Industrial Research, Wellington, New Zealand.

### References

- <sup>1</sup> Taylor, N. H., Soil Bur. Map, 1948, S.B. 280
- <sup>2</sup> *N.Z. Soil Bur.*, 1954, Bull. 5
- <sup>3</sup> Taylor, N. H., *Aust. vet. J.*, 1952, **28**, 183
- <sup>4</sup> Birrell, K. S., & Fieldes, M., *J. Soil Sci.*, 1952, **3**, 156
- <sup>5</sup> Fieldes, M., *N.Z. J. Sci. Tech.*, 1956, **37** (B), 336
- <sup>6</sup> Fieldes, M., & Swindale, L. D., *N.Z. J. Sci. Tech.*, 1954, **36** (B), 140
- <sup>7</sup> Fieldes, M., & Williamson, K. I., *N.Z. J. Sci. Tech.*, 1955, **37** (B), 314
- <sup>8</sup> Fieldes, M., Walker, I. K., & Williams, P. P., *N.Z. J. Sci. Tech.*, 1956, **38** (B), 31
- <sup>9</sup> Wells, N., *N.Z. J. Sci. Tech.*, 1956, **37** (B), 473
- <sup>10</sup> Farmer, V. C., *Spectrochim. Acta*, 1950, **4**, 224
- <sup>11</sup> Mitchell, R. L., *Bur. Soil Sci., Harpenden*, 1948, Tech. Commun. 44
- <sup>12</sup> Wells, N., *N.Z. J. Sci. Tech.*, 1956, **37** (B), 482
- <sup>13</sup> Cunningham, I. J., & Hogan, K. G., *N.Z. J. Sci. Tech.*, 1956, **38** (A), 248
- <sup>14</sup> Fergusson, G. J., & Rafter, T. A., *N.Z. J. Sci. Tech.*, 1955, **37** (B), 371
- <sup>15</sup> Fergusson, G. J., & Rafter, T. A., *N.Z. J. Sci. Tech.*, 1953, **35** (B), 127
- <sup>16</sup> Burns, M. M., & Taylor, N. H., *N.Z. Dep. sci. industr. Res.*, 1939, Bull. 66

### Discussion

*Dr. J. W. S. Reith* (Macauley Institute): Similar survey work is being done at the Macauley Institute. Although the work is not far enough advanced to draw definite conclusions, the results obtained suggest that there are as big variations with copper within any one soil type as between different types. The method has advantages and disadvantages.

*Dr. E. J. Hewitt*: It is generally found that molybdenum deficiency is associated with soil acidity. Could Mr. Fieldes tell me how molybdenum retention by New Zealand soils is affected by pH?

Mr. Fieldes: The affinity of soils for molybdenum is related to adsorption of anions. Soils containing micaceous clay minerals and other colloids in which aluminium is in 6-co-ordination behave differently from soils containing allophane in which it is now thought that aluminium is in 4-co-ordination. The chemistry of the latter is different from that of 6-co-ordinated aluminium, and different also from that of synthetic anion-exchange resins. It hinges about the tetrahedral oxygen at the broken bonds. This site has particular affinity for anions containing oxygen. The series of anion affinities is also understood in terms of the tetrahedral site. In fact pentavalent anions in tetrahedral co-ordination are strongly held because the full valency of oxygen is

satisfied. Thus sorbed phosphates, molybdates and vanadates are stable. When the pH changes, there are two effects: firstly the state of co-ordination of the anions may vary and, secondly, the hydroxyl ion may compete for the site. Changes in molybdate retention with varying pH therefore have a rather complicated pattern, but they do indeed vary and their manner of adsorption and release should be understood in terms of the nature of the site.

*Dr. Hewitt:* Can the valency of molybdenum in soils be usefully studied in relation to pH by using chromatographic methods, as has been done by Stevens?

Mr. Fieldes: Yes.

*Dr. R. L. Mitchell:* What are Mr. Fieldes' conclusions about the efficiency of sweet vernal in indicating soil copper status? At the Macaulay Institute grasses are found to be much less useful than clover for this purpose.

Mr. Fieldes: Smooth curves are obtained for the molybdenum content of grass grown on soils within a suite, and we are therefore satisfied with the data even though the differences are not large when considered absolutely.

## TRACE-ELEMENT UPTAKE IN RELATION TO SOIL CONTENT\*

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Numerous factors affect the uptake of trace elements by plants and lead to variations in the distribution within the plants themselves. All serve to complicate the diagnostic assessment of the trace-element status of a soil by plant analysis. In Scottish soils the most important pedological factor influencing trace-element availability is the drainage status. Under poor drainage conditions the extractable contents of many trace elements in soils are increased, and in certain instances, notably cobalt and nickel, there is a corresponding increase in plant uptake. The relative contents of trace elements in the constituent species of a mixed herbage are not constant; in one mixed herbage, cocksfoot contained the highest cobalt content. Several examples of the effect of soil treatment on plant content of trace elements are quoted, illustrating in particular the difference between different species in their response to soil additions. The use of acetic acid and ethylene diaminetetra-acetic acid (EDTA) as extractants for the diagnostic assessment of the copper and cobalt status of soils is illustrated by results from some 50 soils of various types. There is some indication that EDTA may be better than acetic acid for these elements.

**Introduction**

Our knowledge of the many factors involved in the correlation of plant uptake of trace elements with soil content is far from complete. In this short communication attention is directed to a few of the soil conditions which influence uptake, and, for a few of the biologically important trace elements, our findings on the diagnostic assessment of soil status in relation to plant uptake are summarized.

It is not possible in the space available to discuss variations in plant content due to growth or climatic factors, or changes in distribution within the plant itself at different stages of maturity. Nor is it possible to do more than mention differences in the uptake of different species in so far as they affect assessment of availability. In view of the changes in the composition of plant samples which can arise at different times of sampling, there will probably be general agreement that, once the factors involved are appreciated, the most satisfactory long-term picture of trace-element status of the vegetation carried by any particular soil should be obtainable from examination of the soil itself. The investigations reported here have been carried out with this objective.

The levels of constituent species of the hay crop sampled at flowering in early summer have been selected as a criterion of plant composition against which to judge composition. Investigations in progress have demonstrated the effects of agricultural practice on plant composition, as for instance the difference between pasture herbage regularly cut and that allowed to grow to maturity. One objection to sampling a grazed herbage is the difficulty of avoiding soil contamination, and a second that it is what the animal has rejected that is available for sampling.

Plant uptake cannot be related directly to the total trace-element content of a soil. Even in the most acute instances of deficiency there is more than enough of the element in the soil to provide adequately for the crop if it were all readily available. Although a pasture herbage containing sufficient cobalt to sustain ruminants in a healthy condition need reduce the cobalt content of the surface soil by no more than 0.0005 p.p.m., deficient herbage occurs on soils containing several parts per million total cobalt. Total content may, however, provide diagnostic indication of trace-element status by confirming or ruling out the possibility of excess of such elements as zinc or nickel.

The results recorded here indicate some findings in the course of investigations into the effect of soil treatment on plant uptake and into the efficiency of various soil extractants for diagnostic purposes. The factors affecting plant uptake of trace elements include, apart from total content, the forms in which they occur in soils, the prevailing soil conditions and the characteristics of the plant species concerned.

\* Read at Symposium on Trace Elements in Soils, Plants and Animals, organized by the Agriculture Group and Bristol Section, 10-12 April, 1957

All the trace-element determinations have been made by spectrochemical methods, employing techniques already described.<sup>1-5</sup> Soil extractions reported here have been made with 0.5N-acetic acid (2.5%, pH 2.5) by the procedure detailed in the above references and also with 0.05M-ethylenediaminetetra-acetic acid (EDTA) solution neutralized to pH 7 with ammonia.<sup>6,7</sup> In this instance 10 g. of soil are shaken for one hour with 50 ml. of extractant. After filtration, the extract is evaporated to dryness in silica and the organic material destroyed by ashing. The residue, after treatment with HCl, is taken up in dilute HNO<sub>3</sub> for the determination of Cu by the porous-cup solution spark technique in one aliquot, and of the other elements by the concentration method<sup>2,5</sup> in a second aliquot after removal of nitrate.

### The effect of drainage conditions

Among the many pedological factors which can influence the mobilization of trace elements in soils, the most important so far found in Scottish conditions is the drainage status. The increased ease of extraction of many trace elements from the lower, gleyed layers of a poorly drained soil compared with those at corresponding depths in a well drained soil on the same parent material has already been reported.<sup>8</sup> Hill, Toth & Bear<sup>9</sup> have suggested that the cobalt content of plants growing on poorly drained soils is greater than on well drained soils. Similar effects have been found for cobalt and other elements in poorly drained areas in Scotland, and are illustrated by the results in Tables I and II, which present analyses of soil extracts and plant materials from a soil on an argillaceous schist. An area of poor drainage was so situated that first-year herbage samples were available from well and poorly drained sites in both 1953 and 1954. Apart from drainage, no major differences in the soils of the wet and dry portions are apparent.

Table I

Extractable trace elements (p.p.m. in air-dry soil) in soils from well drained and poorly drained areas of adjoining fields sampled in 1953 and 1954

		Co	Ni	Mo	Fe	Zn	V	Ti	Cr	Cu	Mn	Pb	pH
<i>Acetic acid soluble</i>													
1953	Well drained	1.3	1.3	0.04	<100	5.5	0.7	0.5	0.35	0.17		1.3	5.8
	Poorly drained	2.9	3.4	0.05	<100	6.3	1.7	0.9	0.36	1.11		<1	5.9
1954	Well drained	1.0	1.0	0.04	<100	3.5	0.3	<0.7	0.46	0.13		<1	6.4
	Poorly drained	2.7	5.6	0.06	<100	6.3	1.0	0.5	0.24	0.98		1.3	6.1
<i>EDTA extractable</i>													
1953	Well drained	0.38	1.1	0.06	350	4.3	2.2	3.8	0.47	2.6	13.6	5.4	5.8
	Poorly drained	1.8	4.2	0.19	900	<10	3.4	9.6	1.3	6.6	8.4	7.6	5.9
1954	Well drained	0.27	1.1	0.07	300	3.8	0.5	3.3	0.58	1.7	7.0	6.7	6.4
	Poorly drained	2.0	4.1	0.14	950	<10	1.7	10.2	1.4	6.8	10.2	7.5	6.1

In Table I are recorded the amounts of various trace elements extracted by acetic acid and EDTA. Unusually large amounts of cobalt, nickel, vanadium and copper are extracted by both reagents from the poorly drained soils, and the same applies to molybdenum, iron, titanium and chromium in the EDTA extractions. Comparison of the degree of removal by the two reagents is interesting: amounts of zinc and nickel are similar in both extractants, but EDTA takes out less cobalt than does acetic acid, while for most other elements the reverse is the case, with up to ten times as much copper, lead, iron and titanium in EDTA as in acetic acid. These differences between the extractants are in agreement with results from other Scottish soils, and are probably related to the stability constants of the respective chelates on the one hand, and to hydrogen-ion exchange on the other. The drainage effects presumably arise from differences in the rates of weathering, notably of ferromagnesium minerals, in the types of clay mineral formed, and in the nature of the secondary trace-element compounds, among which the organo-metallic complexes are probably of vital importance.

The uptake of cobalt and nickel by the vegetation (Table II) is parallel to the extractable amount in the soil. There are relatively large contents of these elements in the plants growing on the poorly drained areas. On the other hand, the large differences in EDTA-extractable iron between well and poorly drained areas are not reflected in the plant uptake. In the

Table II

Trace elements (p.p.m. in oven-dry material) in vegetation from well drained and poorly drained portions of adjoining fields sampled in 1953 and 1954

		Co	Ni	Mo	Fe	Zn	V	Ti	Cr	Cu	Mn	Ba	Sr
<i>Mixed pasture herbage</i>													
1953	Well drained	0.18	1.4	0.8	54	32	0.09	4.0	0.16	5.4	108	11	26
	Poorly drained	1.4	3.9	1.4	55	31	0.10	4.0	0.19	5.8	122	13	22
1954	Well drained	0.12	0.9	1.5	48	24	0.08	3.0	0.19	3.6	47	10	13
	Poorly drained	0.86	2.3	1.7	53	26	0.09	2.8	0.19	4.1	134	17	12
<i>Rye-grass</i>													
1953	Well drained	0.18	1.0	0.7	34	27	0.08	2.8	0.16	4.0	88	10	12
	Poorly drained	1.5	3.4	1.2	47	29	0.05	2.0	0.12	3.4	116	12	11
1954	Well drained	0.11	0.9	1.3	28	25	0.06	1.8	0.17	3.2	62	11	12
	Poorly drained	0.64	2.1	1.0	44	21	0.05	2.3	0.16	2.6	92	13	10
<i>Red clover</i>													
1953	Well drained	0.16	2.0	1.0	69	38	0.08	4.0	0.14	7.9	39	10	57
	Poorly drained	1.4	5.9	3.1	78	38	0.08	1.3	0.15	10.3	51	14	48
1954	Well drained	0.17	1.0	2.0	79	35	0.05	1.8	0.13	8.9	33	12	39
	Poorly drained	1.2	3.0	2.4	77	37	0.04	2.3	0.13	10.2	46	24	68
<i>Cocksfoot</i>													
1954	Well drained	0.13	0.9	1.2	50	22	0.08	3.8	0.19	4.3	70	6	7
	Poorly drained	0.73	2.4	1.4	43	18	0.07	2.8	0.20	4.8	182	7	7

1953 samples the molybdenum contents of the plants follow the soil values; those for 1954 do not, possibly because of the higher pH values of the more heavily limed soils examined in that year. Differences in uptake of vanadium, titanium and chromium do not occur despite differences in extractable contents in the soils. The only effect with copper is a small increase in clover grown on the wet soil; this finding is in line with results to be presented later.

These effects of impeded drainage are significant both in their relation to the diagnostic assessment of soil status and to the practical agricultural implications, especially with regard to cobalt deficiency of ruminants. The occurrence of nickel toxicity in some areas in north-east Scotland,<sup>10, 11</sup> first reported in 1940 and since studied intensively from the plant physiological aspect,<sup>12, 13</sup> can be ascribed to increased availability resulting from impeded drainage at some stage in the development of the soils concerned.

#### Variations in plant uptake

In general, for the constituents of a mixed herbage, the relative contents of whole plant samples grown on different soils remain reasonably constant. For instance, the clovers are generally higher in cobalt than the grasses, of which timothy shows the lowest content. Differences in stage of maturity may affect the relative contributions somewhat, but seldom enough to change the order appreciably. That there is not always this relationship is shown by the following figures (p.p.m. in oven-dry material) for the cobalt content of herbage from four soils:

	A	B	C	D
Mixed	0.04	0.17	0.10	0.26
Clover	0.08	0.35	0.19	0.18
Rye-grass	0.04	0.11	0.08	0.06
Cocksfoot	0.03	0.12	0.09	0.36

The pasture species from soils A, B and C display a normal distribution, with the content in clover two to three times that of the grasses, but those from soil D have an abnormally high cobalt content in cocksfoot and, in consequence, in the mixed pasture. The results were so abnormal that the analyses and the botanical identification have been confirmed, as an error at sampling or in the laboratory appeared probable. Samples C came from a field adjoining D sampled in the following year, and only the cocksfoot shows an appreciable change. There is an indication of the same effect for nickel, but no such abnormality has been observed for iron, copper or any of the other trace elements in these samples.



The explanation may lie in the occurrence of very marked concentration of many trace elements in the spikelets at flowering and seed formation. Very rapid changes in distribution within the plant are occurring at this period, and these samples may have been taken at a critical time. Several examples of a contrary effect in mixed herbage, namely a marked drop in cobalt content in one of a series of samples taken during spring and early summer, have been met, but here again the precise explanation has not been found, although it is presumably related to flush of growth in at least one of the constituent species. Both effects illustrate the dangers of using a single plant sample to assess soil, or long-term plant, status.

### The effect of soil treatment

In the assessment of soil status the form of occurrence of trace elements in the soil is of vital importance. The most marked contrast with naturally occurring constituents and the most readily studied effect is that produced by the addition of easily soluble compounds. Numerous field experiments have been carried out to study the effect of such additions. The results presented in Table III relate to samples taken in 1953 from plots treated in 1952 or 1953 with trace elements and lime in amounts near the upper limits likely to be used in agricultural practice. The soil in question is a slate of the Foudland Association in Aberdeenshire on which restricted development in clover occurs, but where molybdenum deficiency does not appear to be involved.

Table III

Effect of soil treatment on the trace-element content of vegetation grown on plots treated in April, 1952, or March, 1953, and sampled in June, 1953, expressed as p.p.m. of oven-dry material

	Mo		Zn		Cu		Mn	
	1952	1953	1952	1953	1952	1953	1952	1953
<i>Rye-grass</i>								
Untreated	0.9	0.8	24	29	2.9	3.1	76	76
Ca	1.2	1.1	19	28	2.9	3.0	49	54
Mo + Cu + Zn	1.5	2.0	24	29	3.2	3.4	81	97
Mo + Cu + Zn + Ca	1.9	3.4	23	26	3.1	3.1	52	65
<i>Red clover</i>								
Untreated	2.0	0.7	33	33	4.7	3.0	49	36
Ca	9.3	3.4	27	29	6.8	4.3	45	35
Mo + Cu + Zn	11.4	18.6	47	75	8.8	7.0	43	49
Mo + Cu + Zn + Ca	16.7	35.6	33	44	7.7	6.2	39	26

The amounts applied per acre were:

Molybdenum	1 lb. of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$
Copper	20 lb. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
Zinc	20 lb. of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
Lime	3 tons of $\text{CaCO}_3$

In 1952, treatments were applied before sowing the oat cover crop; in 1953 the young grass was top-dressed. Lime was applied a few weeks earlier than the trace elements in both years.

In the first place, attention must be drawn to the increased uptake of molybdenum by clover, particularly in association with lime. The amount of molybdenum added, approximately 0.2 p.p.m. to the surface soil, is of the same order as the EDTA-extractable molybdenum in the poorly drained soils previously considered (Table I). The greater availability of the added molybdenum is obvious from the much greater contents in the clover in Table III compared with that in Table II. The effect of liming, which brought about only a relatively small change in soil pH from 6.0 to 6.4, on the uptake of molybdenum must be emphasized, in view of the possible dangers of indiscriminate addition of trace elements, and of molybdenum in particular, to soils which do not require them. In Scotland many soils are near the molybdenum excess level, and animal disorders may well arise if unnecessary additions are made. A similar warning has recently been given by Cunningham & Hogan<sup>14</sup> for New Zealand soils. No trace-element addition should be made to a soil unless its desirability for the crop in question has been established. Shotgun, or what are perhaps better termed blunderbuss, mixtures of trace elements may eventually produce unfortunate consequences.

Little or no effect can be observed (Table III) on the uptake of copper or zinc by rye-grass following treatment with these elements, but the contents in clover are considerably increased. In clover the uptake of zinc is reduced after liming, although for manganese it is rye-grass which shows the greater effect of liming. The effect of lime on the uptake of cobalt and nickel is well established and need not be considered here. The amounts of copper and zinc added correspond to approximately 2.5 p.p.m. in the surface soil, of the same order as the amounts extracted by EDTA from normal Scottish soils. The amount of copper extracted by acetic acid is generally appreciably lower.

In Table IV are presented results demonstrating the effect of copper and cobalt additions to the soil on the uptake by pasture plants on an Old Red Sandstone soil from Aberdeenshire which is deficient in both elements. The amounts added correspond to 5 p.p.m. of Cu and 0.4 p.p.m. of Co in the surface soil. In the results for copper, which have already been reported,<sup>6</sup> a marked difference in response between grasses and clover is again apparent, a six-fold increase in the clover contrasting with a 30% increase in the grasses. For cobalt the difference is not so marked, the relative increases in all plants being much greater. It has been found difficult under Scottish conditions to increase the copper content of rye-grass above 5 p.p.m. by soil treatment, and an examination of rye-grass separated from over 50 typical herbage on various soil types from north-east Scotland has shown contents below this value, although the contents in clover varied from below 2 to 12 p.p.m. Once again it should be noted that all analyses refer to samples taken from the hay crop at the time of flowering in early summer.

Table IV

*Cobalt and copper contents of mixed pasture herbage and constituent species from duplicated series of replicate plots top-dressed with 1.5 lb. of  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$  per acre and 20 lb. of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  per acre in April, 1954, and April, 1955, and sampled in June, 1955, as p.p.m. in oven-dry material*

<i>Cobalt</i>	Untreated		Cobalt-treated		Factor of increase
	Mixed pasture	0.06	0.06	0.62	
Rye-grass	0.06	0.05	0.56	0.73	12
Red clover	0.04	0.06	0.87	1.10	20
Cocksfoot	0.06	0.05	0.59	0.68	12
Crested dogstail	0.05	0.06	0.59	0.72	12
Timothy	0.03	0.03	0.25	0.40	11

<i>Copper</i>	Untreated		Copper-treated		Factor of increase
	Mixed pasture	3.4	2.8	5.6	
Rye-grass	2.4	2.7	3.2	3.3	1.3
Red clover	1.4	1.3	7.8	8.4	6.0
Cocksfoot	3.5	2.8	4.0	4.1	1.3
Crested dogstail	2.8	2.9	3.7	3.8	1.3
Timothy	2.7	2.7	3.4	3.4	1.3

The results in Fig. 1 for incremental cobalt treatments of a granitic soil two years after treatment with 0, 2, 10 and 40 lb. of  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  per acre (0.0, 0.25, 1.25 and 5 p.p.m. Co to the surface soil) show the plant uptake plotted against acetic-acid-soluble cobalt in the soil, which initially contained 0.47 p.p.m. of soluble cobalt. It will be seen that much of the added cobalt has remained acetic-acid-soluble after a lapse of two years. The grasses show a smaller uptake than red clover, particularly for the largest addition. The clover uptake is almost linear and cuts the axis at a point corresponding to 0.40 p.p.m. of acetic-acid-soluble cobalt. This would suggest that 0.47 p.p.m. of extractable cobalt in this soil in its natural form is equivalent to 0.07 p.p.m. added as cobalt chloride, in so far as uptake by clover is concerned. One consequence of this difference in effectiveness is to make diagnosis by an extractant such as acetic acid very uncertain with soils that have received prior cobalt treatment. An extractant giving a curve passing nearer to the origin, i.e., taking out less of the naturally occurring soil cobalt, would be preferable.

#### The assessment of soil status

The correlation between plant uptake and extractable soil-copper, in relation to the incidence of copper deficiency in oats, has been considered in a previous paper,<sup>6</sup> which discusses results

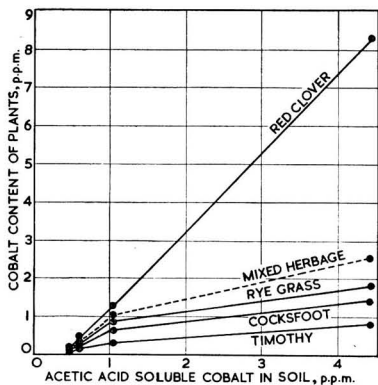


FIG. 1.—Relationship between plant content and acetic-acid-soluble soil cobalt from plots on a granitic soil which had received incremental cobalt treatments

from some 50 soils, sampled in 1953, 1954 and 1955, of the various types which occur in north-east Scotland. It was found that there is a reasonable correlation between EDTA-extractable copper and the copper content of clover at low copper contents, but that the content in the plant did not rise above 12 p.p.m. even at high copper contents of the soil. Copper deficiency in oats occurred on soils which carried clover with less than 4 p.p.m. of copper, and whose EDTA-extractable copper value (which ranged from 0.37 to 6.8 p.p.m.) was approximately 1 p.p.m. or less. It could not be diagnosed by analysis of the oats. There was little or no correlation between the copper content of rye-grass and EDTA-extractable copper. Figs. 2 and 3 summarize the results obtained and include data for additional soils examined in 1956. The slope of the mean curve for clover is very steep until a content of 8 p.p.m. is reached, but appears to pass through the origin. It is considered wise at present to recommend copper additions to all soils with EDTA-extractable copper below 1.5 p.p.m. if there is any suggestion of plant or animal disorder.

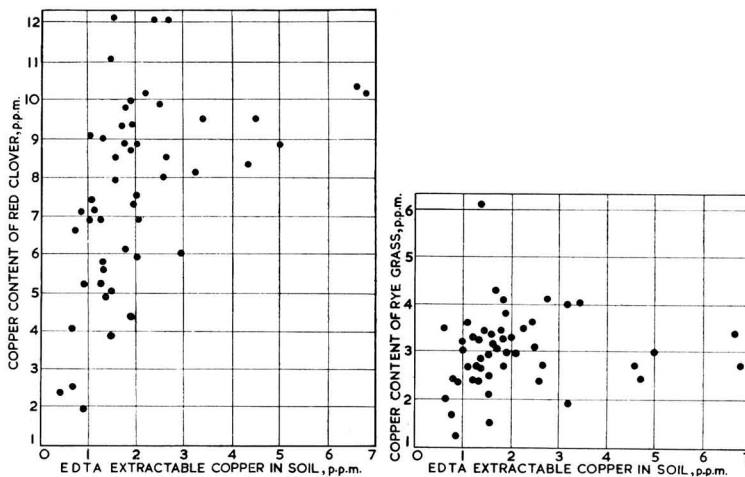


FIG. 2.—Relationship between the copper content of red clover and the amount of EDTA-extractable copper in soil  
 FIG. 3.—Relationship between the copper content of rye-grass and the amount of EDTA-extractable copper in soil

High contents of extractable copper have generally been found in poorly drained soils, but the clover maximum at about 12 p.p.m. cannot be attributed to a restriction due to a drainage factor, as addition of 7.5 p.p.m. of copper in soluble form to a freely drained soil is ineffective in raising the clover content above this level. There appears to be some physiological control of luxury uptake.

The results for acetic-acid-soluble copper in soil are much less satisfactory, the highest clover copper occurring on a soil which has very low acetic acid but high EDTA-extractable copper. The range of acetic-acid-soluble copper is from 0.02 to 1.1 p.p.m., considerably lower than that for EDTA, in agreement with the results in Table I. It appears probable that while acetic acid extracts a fraction of the exchangeable or inorganically bound copper, EDTA can also extract some organically complexed copper, including that fraction which is available to plants. The amount of copper removed from a surface soil by a 5-ton crop containing 10 p.p.m. of copper is approximately 0.05 p.p.m., which is greater than the amount extracted from many soils by acetic acid.

Determinations of other trace elements have been made on the same samples. The most interesting results are those for cobalt. A statistical examination has shown a highly significant ( $P < 0.001$ ) correlation coefficient for both EDTA and acetic acid extractions against mixed herbage, rye-grass and clover contents. Graphical presentation suggested that, under the experimental conditions employed, the best correlation at low cobalt contents is between cobalt content of rye-grass and EDTA-extractable cobalt in the soil (Fig. 4), and statistical analysis confirms this. In practice it is the content in the mixed herbage which is of importance, and in view of the analytical convenience of acetic acid compared with EDTA in so far as the extraction of cobalt is concerned, the results for this reagent are presented in Fig. 5. Increased scatter for mixed herbage compared with rye-grass is inevitable because of variations in the ratios of the different species at different locations. This effect is possibly more marked at high than at low cobalt contents (cf. Table IV and Fig. 1). The borderline is placed at about 0.3 p.p.m. of acetic-acid-soluble cobalt in mineral soils with a pH value about 5.5. Taking 0.08 p.p.m. of cobalt in the herbage as the criterion of cobalt deficiency (although this value is probably rather high for a herbage allowed to grow to maturity) it will be noted that a few soils with acetic-acid-extractable cobalt values above 0.3 p.p.m. fall into the low category and reduce

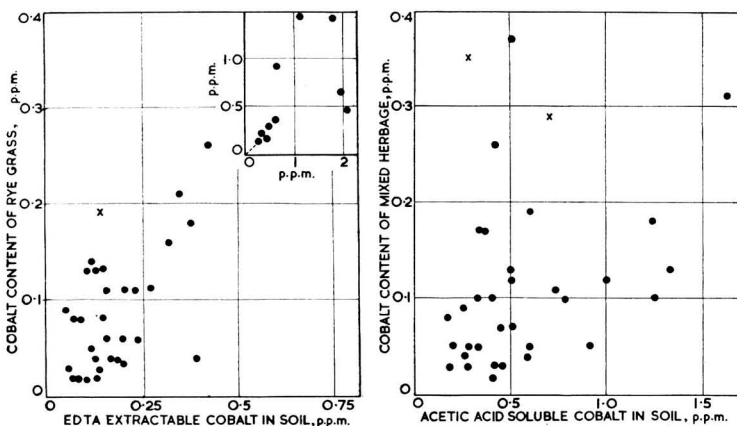


FIG. 4.—Relationship between the cobalt content of rye-grass and the amount of EDTA-extractable cobalt in soil  
 × indicates that the soil is known to have received Co treatment

FIG. 5.—Relationship between the cobalt content of mixed herbage and the amount of acetic-acid-soluble cobalt in soil  
 × indicates that the soil is known to have received Co treatment

the significance of the correlation. In every instance except one the soil has a pH value above 6. The exception has 0.32 p.p.m. of extractable cobalt and is in fact the soil which in Fig. 4 shows a very abnormal EDTA relationship (0.04 p.p.m. of cobalt in rye-grass : 0.39 p.p.m. of EDTA-extractable cobalt).

For both extractants a number of plant contents are somewhat higher than one might expect from the soil values. Those marked by a cross in Figs. 4 and 5 are known to have received cobalt treatment, and it is quite possible that other soils have received some cobalt, as it is now very difficult to determine precise fertilizer histories of specific fields in areas where cobalt deficiency is known and additions may have been made without consultation with the advisory services.

It may be that the decreased scatter with EDTA extraction is related to the fact that it is a less highly buffered reagent, with the pH value of the soil influencing the extraction equilibrium.

The EDTA-extractable cobalt in soil is generally appreciably less than the acetic-acid-soluble, and it seems unlikely that, in the mineral soils concerned, organically complexed cobalt is important. The stability constant of the EDTA-cobalt complex is smaller than that for copper, but it is improbable that this can account fully for the difference in behaviour between cobalt and copper with the two extractants.

### Conclusion

The foregoing sections present a brief progress report on a series of long-term investigations on soil-plant relationships. The effects of drainage status and soil treatment are of obvious significance in determining plant uptake, but other factors as yet not clearly defined can influence this. Investigations at present in progress at the Macaulay Institute suggest that the nature of the organic complexes which occur in soils are of vital importance as far as trace-element uptake is concerned. The ultimate aim, from the diagnostic point of view, is to find an extractant which will indicate the soil status, by means of a single-value soil determination, for soils of all types. The results so far obtained suggest that EDTA extraction is a promising tool for the assessment of copper status, and is possibly also superior to acetic acid for the determination of the cobalt status.

### Acknowledgment

It is desired to acknowledge the valuable assistance of Mr. J. R. Devine in much of the field work and of Mr. R. H. E. Inkson for the statistical evaluation of the results.

### References

- <sup>1</sup> Mitchell, R. L., 'Spectrographic Analysis of Soils, Plants and Related Materials', *Bur. Soil Sci., Harpenden*, 1948, Tech. Commun. No. 44
- <sup>2</sup> Mitchell, R. L., 'Spectrochemical Determination of Trace Elements in Plants and Other Biological Materials', *Proc. Symp. on Trace Analysis*, in press (New York: Wiley)
- <sup>3</sup> Mitchell, R. L., *Proc. Soil Sci. Soc. Fla.*, 1955, **15**, 12
- <sup>4</sup> Mitchell, R. L., & Scott, R. O., *Applied Spectroscopy*, 1957, in press
- <sup>5</sup> Mitchell, R. L., & Scott, R. O., *Spectrochim. Acta*, 1948, **3**, 367
- <sup>6</sup> Mitchell, R. L., Reith, J. W. S., & Johnston, Isabel M., 'Soil Copper Status and Plant Uptake', *Analyse des Plantes et Problèmes des Fumures Minérales, Travaux du Colloque à l'Occasion du VI Congrès Intern. Sci. Sol, Paris*, 1956, in press
- <sup>7</sup> Viro, P. J., *Soil Sci.*, 1955, **79**, 459
- <sup>8</sup> Mitchell, R. L., 'Trace Elements' in 'Chemistry of the Soil', Amer. Chem. Soc. Monograph No. 126, 1955, p. 253 (New York: Reinhold)
- <sup>9</sup> Hill, A. C., Toth, S. J., & Bear, F. E., *Soil Sci.*, 1953, **76**, 273
- <sup>10</sup> *Annu. Rep., Macaulay Inst. for Soil Research*, 1940-41
- <sup>11</sup> Mitchell, R. L., *Proc. Nutr. Soc.*, 1944, **1**, 183
- <sup>12</sup> Hunter, J. G., & Vergnano, O., *Ann. appl. Biol.*, 1952, **39**, 279
- <sup>13</sup> Crooke, W. M., *Ann. appl. Bot.*, 1955, **43**, 465
- <sup>14</sup> Cunningham, I. J., & Hogan, K. G., *N.Z. J. Sci. Tech.*, 1956, **38** (A), 248

**Discussion**

*Dr. F. E. Moon* (Fisons) : Is Dr. Mitchell of the opinion that, for assessment of trace-element level (particularly of cobalt), soil analysis is superior to herbage analysis, and if so, is this level of assessment equally applicable to (a) grass repeatedly grazed or defoliated at an immature stage, and (b) grass utilized at a more advanced stage of maturity (e.g., hay or silage) ?

*Dr. Mitchell* : With cobalt it is necessary to find out what an animal has eaten or is going to eat : the obvious method would be to analyse the herbage at various times, but this would entail an impracticable amount of work. We are trying to find a method of soil analysis which would assess the mean herbage content over the growing season. In an experiment at present in progress near Aberdeen cuts are being taken at 2-3-week intervals and the constituent plants broken up into their separate parts. Some 900 samples are being analysed for some 20 elements. When this work is completed, there will be a better answer to the question, but it will apply only to that particular soil. Regularly cut pasture may have up to twice the cobalt content of one approaching maturity ; as the maturing sample ripens in August and September certain elements may rise appreciably.

*Dr. A. M. Smith* (Edinburgh) : There is little doubt about the difficulties in getting a representative sample of herbage for analysis and assessment, but there are also large variations in the soil and great care is required to obtain a reliable soil sample.

*Dr. Mitchell* : A method of representative soil sampling would deal with this problem. For areas up to four acres it is necessary to take 20/30 borings, which are combined to give a representative sample.

*Prof. Wallace* : Dr. Mitchell has made an excellent case for examining both herbage and soil : in sampling soils for molybdenum deficiency Dr. Plant has sometimes had to sample underneath the roots of deficient plants to show the deficiency. It is well known that to obtain valid comparisons similar physiological stages of plant material must be examined. It is also well known that there are large differences between species and that if soil analysis is relied upon to indicate toxicity in pastures, it is essential to know their botanical composition. On teart soils in Somerset, clovers have a much higher content of molybdenum than grasses. Farmers cannot, therefore, improve pastures by means of clover because of the danger of raising the molybdenum content. Yorkshire Fog is a high-molybdenum grass and may also be injurious. Variations in both soil and herbage must be taken into account. With pastures one hopes that the animal will eat the grass and not the soil.

*Dr. Mitchell* : As an example of distribution within the mature plant, the greatest proportion of certain elements, such as zinc, may be found in the inflorescence. The bulk of other trace elements may occur in the leaves or in the stems.

*Dr. van Erkelens* : Is Dr. Mitchell familiar with experiments indicating that, as expected by most workers in this field, the cobalt in the soil is not available to the animal ?

*Dr. Mitchell* : Yes ; the bulk of the cobalt in soil is not readily extractable but is actually a part of the crystal structure of the ferromagnesium minerals, replacing ferrous iron or magnesium. It is unlikely to be brought into solution in the digestive tract.

*Dr. E. C. Owen* : Is cobalt extractable from ferromagnesium minerals by EDTA ?

*Dr. Mitchell* : This is not possible until there has been some weathering of the minerals.

## SOME NUTRIENT INTERACTIONS AFFECTING THE GROWTH OF PASTURE LEGUMES IN ACID SOILS\*

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### Introduction

The effect of metallic ions on the growth of plants has received considerable attention over the last twenty-five years and there is no need to review the extensive literature on the subject. It is sufficient to point out that whilst those elements required in large quantities, such as calcium and potassium, are never present at toxic levels, those elements of which the plant requires only trace quantities (the micro-nutrients)—notably manganese, copper, and zinc—give rise to striking symptoms at both deficiency and toxicity levels. These deficiency or toxicity symptoms are accompanied by reductions in growth *rate* and in the subsequent yield of the crops produced.

Between the levels of supply corresponding to deficiency or toxicity symptoms lie those permitting optimum growth. These levels are not fixed, even for one plant species, and it has become increasingly obvious that considerable interaction between the nutrients may take place. That is to say, a level of supply of a nutrient which produces optimum growth with one group of other nutrients at a certain level may lead to deficiency or even to toxicity should the level of one of these others be varied considerably. The availability of the micro-nutrients to the plant has been found to vary widely with the acidity of the soil. This effect is particularly marked with molybdenum and manganese. For molybdenum the availability falls drastically at high acidity, whereas for manganese the change is in the opposite direction and manganese deficiency has most commonly been associated with alkaline soils. The work of Hewitt and his colleagues at this Station in showing that the deleterious effects of acid soils are due as much to the toxic effects of manganese and aluminium as they are to deficiency of calcium or to the acidity itself is consequently of particular interest. Theoretically a situation could arise where merely by change in the pH of the soil the availability of manganese would change from a deficiency to a toxicity level or *vice versa*.

From this work it has become obvious that the effects of soil acidity on plant growth are due largely to the variations in availability of certain nutrients associated with variations in the acidity. These effects can in fact be subdivided into: (a) a deficiency of soil calcium or magnesium, (b) a deficiency of molybdenum due to lowered availability, (c) an excess of manganese or aluminium (and possibly nickel and chromium also), (d) a deficiency of available phosphate due to the high anion-exchange capacity produced by dissociation of hydroxyl ions, liberated mainly from the sesquioxides and to a lesser extent from the clay minerals, and (e) a direct effect of the pH on the growth either of the plant or on the soil micro-organisms.

Consequently it seemed of interest to explore the extent to which variations in levels of micro-nutrients, particularly those whose availability was affected by variations in acidity, might interact on the growth of pasture legumes. Two aspects were of particular interest. In the first place, the pasture legumes include species of widely varying tolerance to soil acidity, ranging from lupins which may readily show lime-induced chlorosis to lucerne and sainfoin which are always associated with chalk soils or heavy liming, and knowledge was required about the mechanism of these differences. In the second place, although deficiencies sufficiently marked to give clinical symptoms were found rarely in nature, it seemed probable that in many cases the level of supply of a nutrient would be low enough seriously to restrict growth. To such a condition of restricted growth, without the development of characteristic lesions, the term sub-clinical deficiency, applied elsewhere, has been adopted here.

### Interactions between low levels of nutrients and toxic elements

The effect of increased levels of manganese and aluminium on lucerne (*Medicago sativa* L.), white clover (*Trifolium repens* L.) and lupin (*Lupinus luteus* L.), in both sand culture and

\* Read at Symposium on Trace Elements in Soils, Plants and Animals, organized by the Agriculture Group and Bristol Section, 10–12 April, 1957

water culture, was examined and certain points of difference between these species were found. It was also possible to differentiate between the effects of a low calcium supply and the toxic effects of high levels of manganese and aluminium. These effects, however, are not unrelated and the calcium level has a distinctly moderating influence on the effects of both manganese and aluminium.

For clover and lucerne very low calcium levels (1.0 p.p.m.) lead to drastically reduced growth, even at low concentrations of manganese and aluminium (0.3 p.p.m. of each), but lupin plants were only slightly stunted. Although the plants of the two former species in the low calcium supply were only one-seventh to one-eighth of the size of those receiving the best treatment, the only symptoms of calcium deficiency were a mild and generalized lightening of leaf colour to a yellow-green, whilst the roots were thin and restricted.

Higher levels of aluminium produce definite symptoms in lucerne and clover. In lucerne there is a general stunting of growth and the leaves are paler (not true chlorosis but a lighter green of the middle). The lower leaves show a purple coloration and in some cases the petiole collapses. There is characteristic nipping and withering of the upper leaves, the margin whitening whilst the surface crinkles and is speckled white.

With white clover the symptoms are not so marked. The leaf surface showed blotches and speckles and the margin of the leaf became withered. With lupin, however, far from there being any toxicity symptoms (at 40 p.p.m.) the plants not only looked completely healthy but actually made better growth than those receiving less aluminium.

The effect of aluminium on root growth is also quite distinct. The roots are severely restricted, shorter, squatter and brown. In another set of water culture experiments this effect was found to be more marked, even with such a notably acid-tolerant plant as serradella.

Excess manganese on the other hand produces in lucerne a chlorotic mottling on the margin of the leaflets, the spots spreading towards the centre and coalescing. The leaflets eventually become yellow with brown spots. The marginal withering gives a cupped effect and there is often a reduction in leaf size.

With white clover the effect is less marked and is restricted to a general chlorosis, particularly of the margins, whilst with lupin only a slight chlorosis is seen, taking the form almost of speckling.

All these effects are moderated, as mentioned earlier, by the level of calcium supplied. From many data obtained, those in Table I, showing the interaction between the levels of calcium and aluminium, illustrate this point.

The results show the restrictions of calcium deficiency even at low levels of aluminium, as well as the toxic effects of high levels of aluminium. The highest level of calcium supplied (330 p.p.m.) gave a lower yield at low aluminium level than did a lower level of calcium (67 p.p.m.), owing to an interference with potassium uptake, for these high Ca plants showed distinct signs of potassium deficiency. On the other hand, when 10 p.p.m. of aluminium was being supplied, the 330 p.p.m. of calcium gave the best yield, and the plants showed almost no symptoms

Table I

*Effect of variations in the Ca and Al level on the growth of lucerne in sand culture\* (in g. of dry matter per plant)*

Ca, p.p.m.	Al, p.p.m.			
	0.3	5.0	10.0	40.0
1	1.1	1.7	1.2	—
10	—	—	—	1.36 (2.39)†
67 or 65‡	7.6	4.7	4.0	2.1 (5.19) 2.43 (4.33)
120	—	—	—	2.17 (5.96) 2.33 (4.49)
330	5.6	3.9	4.8	—

\* The plants were grown in acid-washed sand and a standard nutrient solution (Long Ashton) to which were added various concentrations of Ca as  $\text{Ca}(\text{NO}_3)_2$ , Mn as  $\text{MnSO}_4$ , and Al as  $\text{Al}_2(\text{SO}_4)_3$ . The pH was adjusted to 6.0 by additions of 0.1N- $\text{H}_2\text{SO}_4$  or -NaOH. Variations in  $\text{Ca}(\text{NO}_3)_2$  were balanced by  $\text{NaNO}_3$ , so that the nitrate level remained constant at 224 p.p.m. of N.

† The figures at 20 and 40 p.p.m. come from a later experiment, the figures in brackets being those at 10 weeks old, whilst all the others were harvested at 8 weeks.

‡ 67 p.p.m. of Ca was given to those treatments receiving 0.3, 5.0 and 10.0 p.p.m. of Al, and 65 p.p.m. of Ca to the other two treatments.



of aluminium toxicity, one plant showing only a slight nipping of the leaf but no petiole collapse, withering of the leaf or whitening of the margins, whilst the roots were white and healthy.

The plants receiving 5 p.p.m. of aluminium and 67 p.p.m. of calcium were very similar to the last group in appearance and size, but again none of the plants showed any nipping. Those at the same level of calcium but receiving 10 p.p.m. of aluminium showed only one plant with slightly nipped leaves, even though the growth was little more than half that of the plants treated in the same way but receiving the lower level of aluminium. This appears to be a clear case of what might be termed a 'sub-clinical' toxicity.

It might be mentioned here that a direct effect of pH on the growth of both white clover and lucerne was found which is distinct from the influence of manganese and aluminium, but it is operative only at the *germinating seedling* stage.

#### Interactions between low levels of nutrients

Another aspect of the soil acidity problem is the insidious occurrence of single and multiple deficiencies not severe enough to produce clinical symptoms.

Copper is of interest in this respect as in light-textured acid soils it is mobile and may be largely leached out of the profile. Using sand culture and the Mount Barker strain of subterranean clover (*Trifolium subterraneum*) as the test plant, we have found levels of copper supply which may cause a very large reduction in growth both directly and by a strong interaction with molybdenum, even though the growth produced was free from the symptoms of deficiency.

In this case the nutrient solution used was the basic Long Ashton solution but with a calcium level of 64 p.p.m. and  $\text{NO}_3^-$  at 224 p.p.m. of N. The following variables were introduced:

Cu	0.065 p.p.m.*	Mo	0.40 p.p.m.	P	82 p.p.m.
	0.0065		0.04		41
			0.004		8

\* Long Ashton level

The whole was duplicated to provide for a harvest after 10 weeks (8 replicates per treatment) and another after 16 weeks (7 replicates per treatment).

The effect of reducing the Long Ashton level of copper tenfold was remarkable throughout the range of treatments. The mean yields for the high and low copper treatments were 14.5 and 6.1 g. per plant respectively. Fig. 1 shows plants grown under conditions of high and low copper level.

The low-copper plants were free from all symptoms of deficiency other than an increase in red pigment of the leaves. Growth under most treatments was vigorous and would have been considered satisfactory without the contrast of the plants receiving the higher level of

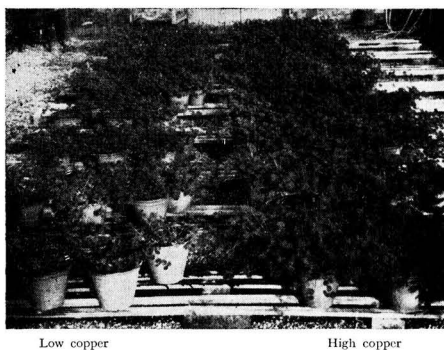


FIG. 1.—Effect of a tenfold reduction of copper on the growth of subterranean clover

copper. In the field the difference would be difficult to detect. The magnitude of the copper effect was confirmed in the following spring (1956), both under the same level of nitrogen, with plants receiving 12 p.p.m. of nitrate N and obtaining the rest of their nitrogen requirement symbiotically, and with plants grown under severe calcium deficiency.

The three variations in molybdenum were tenfold steps. The lowest, 0.004 p.p.m., is probably well above the threshold of deficiency and the highest, 0.4 p.p.m., could not be expected to be toxic. With this in mind and knowing that molybdenum is needed only in minute quantities, no yield differences were expected unless there was some interaction with the other variables. Plants grown with the three levels of molybdenum are shown in Fig. 2.

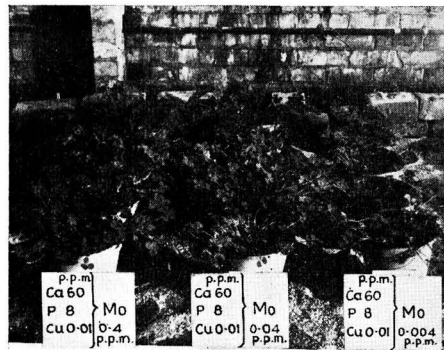


FIG. 2.—Effect of molybdenum on the growth of subterranean clover

No yield differences were obtained, for when copper was present at the higher level (0.065 p.p.m.) there was no significant response to molybdenum. At the lower copper level, however, there was a marked response to molybdenum, the nature of the interaction being modified by the level of phosphorus supplied. The most outstanding treatment differences occurred at 8 p.p.m. of phosphorus. These low-molybdenum plants were miniatures, without any sign of malnutrition. They simply stopped growing about the 10th week. In the field the symptoms would have been impossible to diagnose, but by an increase in the copper supply, a three-fold increase in dry-matter production was obtained.

The complete interaction between Cu, Mo and P at 64 p.p.m. of Ca and 224 p.p.m. of N is set out below in Table II (see also Fig. 3).

It has not yet been possible to get a completely satisfactory repetition of this molybdenum/copper interaction. The interaction was obtained in the first place in the summer and autumn of 1955 when the plants were growing during a period of progressively decreasing day length. The experiment was repeated the following spring at a time of progressively increasing day length. Under these latter conditions the sub-clover passes rapidly through the vegetative to the flowering stage; these conditions cause complications in interpreting results. Inflorescence occurred just before the 13th week and plants were immediately harvested. In the previous autumn the copper/molybdenum interaction was not evident until after the 11th week and did not reach its recorded magnitude until the 15th week. Indications from growth curves taken throughout the 1956 experiment show that the molybdenum treatments were showing differences about the 11th week, and that if extrapolation could be safely made from the 13th to the 15th week, visible differences would have been obtained. However, the low-copper and low-molybdenum treatments did not produce the expected miniatures possibly owing to the different conditions of climate and day length. This experiment will be repeated in 1957.

This last point merely illustrates what has been borne out by other experiments, namely, the necessity for an adequate climatic description to accompany every nutritional investigation.

**Table II**

*Interaction of Mo × Cu × P on subterranean clover*  
 $10 \times \log_{10}$ (mean dry-wt.)

P	p.p.m. Mo	Low copper			High copper		
		Dry wt.	Difference	significant	Dry wt.	Difference	significant
8	0.004	1.489	+ 0.171	5%	2.078	+ 0.036	—
	0.04	1.660	+ 0.165	5%	2.114	+ 0.021	—
	0.4	1.825			2.135		
41	0.004	1.695	+ 0.259	1%	2.223	- 0.014	—
	0.04	1.954	- 0.055	—	2.209	- 0.012	—
	0.4	1.899			2.197		
82	0.004	1.750	+ 0.199	5%	2.155	+ 0.023	—
	0.04	1.949	- 0.086	—	2.178	+ 0.002	—
	0.4	1.863			2.180		

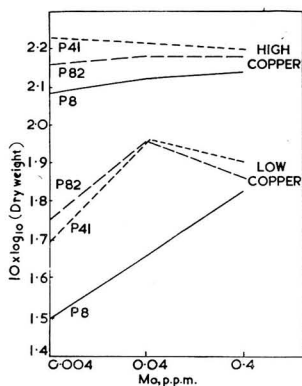


FIG. 3.—Interaction of Mo, Cu and P on subterranean clover

Compare, for example, the lack of symptoms in the miniature plants of the 1955 autumn harvest with the brilliant colours associated with molybdenum deficiency in subterranean clover recently described in Victoria.<sup>1</sup> In the work described here, subterranean clover receiving high levels of phosphate showed a particularly marked necrosis during the high light intensities and high temperatures of the exceptionally sunny May of 1956, but with the dull weather of June the newer leaves produced showed little if any of these symptoms. The same conditioning of the response by climatic factors has been observed with toxic effects also, and even so obvious and easily separable an effect as manganese toxicity in lucerne has been found difficult to produce in a heated glasshouse in late autumn. The extent to which climatic factors influence the rate of growth is probably the main criterion, for the effect of both toxicity and deficiency appear to be most apparent when plants are growing vigorously. With dull weather conditions and the slower growth which commonly accompanies them, it seems not improbable that sub-clinical levels of deficiency or toxicity may occur for a range of elements and their existence be completely unsuspected in the absence of any standard for immediate comparison.

**Reference**

<sup>1</sup> *J. Dep. Agric. Vict.*, 1955, **53**, 456

**Discussion**

*Dr. E. J. Hewitt* : Apart from the effect of calcium on growth at high aluminium levels, has the calcium any effect on root injury?

*Prof. Hallsworth* : There is little effect ; there is some slight improvement in top growth, but adverse effects are still visible.

*Dr. D. J. D. Nicholas* : With reference to the failure to find aluminium in the leaves, large amounts are often found in roots.

*Prof. Hallsworth* : We are proceeding with a complete analysis ; this is the result so far.

*Dr. Williams* (Grassland Research Institute) : Has anything been done on interaction between manganese and potassium ? The pictures of lucerne suffering from manganese toxicity showed leaf symptoms typical of those normally associated with potassium deficiency.

*Prof. Hallsworth* : Analyses have not suggested potassium deficiency except in one or two cases ; these are due not to manganese toxicity but to high calcium content.

*Prof. Wallace* : Has any analysis been done on the miniature plants mentioned by Prof. Hallsworth ?

*Mr. E. A. N. Greenwood* : The miniature plants had a very low copper content of about 1-5 p.p.m. The molybdenum content was similar.

In reply to a further question by *Prof. Wallace*, *Dr. Bould* said that the normal concentration of copper in the dry matter of the leaves of fruit trees was about 5 p.p.m., and that a deficiency level was between 1 and 2 p.p.m. ; if there were sudden stimulation of growth by treating deficient trees with copper, growth might appear normal although the copper content would still be low.

*Prof. Wallace* : Prof. Steenbjerg is an authority on this subject and I should be glad if Mrs. E. Boken would outline his findings for the meeting.

*Mrs. Boken* : Prof. Steenbjerg's work shows that the content of copper in the plant depends on the shape of the yield curve. When the curve relating the percentage nutrient content (ordinate) to the yield (abscissa) is S-shaped, it will at first fall towards the abscissa, pass through a minimum, and then rise. Therefore the highest percentage content may be found when the yield is poorest ; the lowest content occurs at the point of inflexion in the curve. Beyond this point the content of nutrient increases until toxicity sets in.

*Dr. Hewitt* : Dr. Piper and I can confirm Prof. Steenbjerg's experience from work with water and sand cultures. A single analytical level may correspond with either severe deficiency or high yield. I doubt whether an S-shaped curve is always obtained in practice ; if the logarithm of the total nutrient content in the plant ( $\% \times \text{yield}$ ) is plotted against the log of the amount supplied, the result is a straight line.

*Prof. Garner* : Is the physiological reason for this known ?

*Dr. Hewitt* : A trigger mechanism, based on differential requirements for cell expansion following cell division, may be involved.

*Prof. Wallace* : Dr. Hewitt has shown the varying responses made by plants to the individual elements of the acid soil complex, such as low calcium and magnesium, and high aluminium and manganese.

*Dr. Hewitt* : Different plants have different sensitivities, but taking all the elements together one obtains good agreement with the old lists of Arrhenius.

*Prof. Hallsworth* : It is for these reasons that we have studied clover.

*Prof. Wallace* : Some plants, for example tea and some ferns, are accumulators of aluminium and give a different picture—these are among the acid-resistant plants.

## THE COMPLEXING OF COPPER WITH NATURAL COMPONENTS OF FRUIT\*

By **C. F. TIMBERLAKE**

*Long Ashton Research Station, Bristol*

Trace metals normally present in plant and animal tissues may exist both in the free ionic state and in combination with other components. The present account of copper complexes in fruit gives an indication of the type of complex that may be found.

Apples contain traces of copper, zinc and iron, the amounts found in 17 varieties of unsprayed fruit varying from 0.27–0.59 p.p.m. of copper, 0.21–0.76 p.p.m. of zinc and 1.0–2.5 p.p.m. of iron, all expressed as p.p.m. of fresh weight of fruit.<sup>1</sup> In a further seven varieties studied, the metallic content was as follows:

	Fresh weight, p.p.m.	Dry weight, p.p.m.
Copper	0.43–0.66	3.0–4.2
Zinc	0.33–0.57	2.3–3.7
Iron	0.93–3.3	7.0–19

The fruit was washed well with tap water and rinsed with glass-distilled water before analysis. Additional washing of the fruit with dilute acid at several temperatures produced no significant decrease in the metallic content, so that the figures quoted represent the trace metals present as intrinsic biological components and are not due in part to metal traces adhering to the waxy surface of the fruit. The concentrations of copper, zinc and particularly iron are much higher in the peel than in the flesh of the fruit, as shown below.

	Proportion of whole fruit		p.p.m. (dry weight)	
			peel	flesh
Peel	7–17%	Copper	4.1–9.0	2.7–3.7
Copper in peel	14–45%	Zinc	2.6–10.4	1.5–4.1
Zinc in peel	15–36%	Iron	28–91	5–15
Iron in peel	39–60%			

The concentration of these metals in apple peel is of interest since the peel is the source of most of the physiological activity of the apple and also contains one-third of its protein nitrogen.<sup>2</sup>

As these findings would suggest, the copper, zinc and iron of the fruit are only partly soluble in the freshly expressed juice. When rigorous precautions were taken to avoid metallic contamination it was found that juices freshly expressed from three varieties of fruit, contained after centrifuging 24–45% of the total copper in the fruit, 30–50% of the zinc and only 11–14% of the iron. Filtration of the juice through purified paper removed still more of these metals which were associated with finely dispersed tissue particles in the juice. A filtered juice contained only 0.13 p.p.m. iron and 0.20 p.p.m. copper, whereas the fruit from which it was expressed contained 1.6 p.p.m. iron and 0.55 p.p.m. copper (fresh weight). During the large-scale manufacture of apple juice or cider some contamination with metals is possible and freshly expressed juice may contain amounts of metals up to 1 p.p.m. copper and zinc and 3 p.p.m. iron. These levels may change during further processing of the juice.

A known copper-containing constituent of the apple is polyphenol oxidase with a probable copper content of 0.2–0.3%. If all the copper in the fruit were combined in this way, there should be some relation between the copper content of the fruit and its polyphenol oxidase activity. However, initial tests on some apple varieties showed no relationship between the copper and polyphenol oxidase contents of the fruit, and this was confirmed by tests made on the fruit of two varieties sampled from the tree at different stages of growth. Results of analyses of polyphenol oxidase (Ponting & Joslyn<sup>3</sup>) which are expressed in arbitrary units, copper and other fruit components are shown in Tables I and II. Levels of potassium, copper, zinc and

\* Read at Symposium on Trace Elements in Soils, Plants and Animals, organized by the Agriculture Group and Bristol Section, 10–12 April, 1957

Table I

	Changes in apple components based on weight of whole fruit									
	Cu p.p.m.		Zn p.p.m.		Fe p.p.m.		Pb p.p.m.		K	
	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.	Fresh wt. p.p.m.	Dry wt. %
Morgan Sweet 21/6/54	0.77	6.2	1.47	11.8	3.08	24.9	0.20	1.60	1980	1.60
Morgan Sweet 19/8/54	0.42	3.1	0.50	3.7	1.35	10.1	0.03	0.20	1240	0.92
Morgan Sweet 14/10/54	0.33	2.5	0.44	3.3	1.07	8.1	0.11	0.83	1140	0.86
Dabinett 28/6/54	0.53	3.3	1.40	8.8	2.50	15.6	0.01	0.06	2520	1.57
Dabinett 23/8/54	0.26	1.8	0.56	3.8	1.23	8.3	0.07	0.47	1470	0.99
Dabinett 8/11/54	0.25	1.6	0.58	3.6	1.80	11.2	0.12	0.74	1350	0.84
	Na p.p.m.		Total phenols %		Enzyme activity		Total N %		Protein N %	
	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.	Fresh wt.	Dry wt.
Morgan Sweet 21/6/54	26	210	1.41	11.4	350	2830	0.150	1.21	0.128	1.04
Morgan Sweet 19/8/54	45	336	0.44	3.04	5.6	42	0.053	0.40	0.041	0.30
Morgan Sweet 14/10/54	61	463	0.31	2.36	48	365	0.046	0.35	0.034	0.26
Dabinett 28/6/54	19	119	2.19	13.7	527	3300	0.231	1.44	0.173	1.08
Dabinett 23/8/54	26	175	0.65	4.37	26	175	0.075	0.51	0.050	0.34
Dabinett 8/11/54	63	391	0.46	2.86	44	273	0.052	0.32	0.039	0.24

Table II

Variety	Changes in apple components per fruit									
	Cu $\mu$ g.	Zn $\mu$ g.	Fe $\mu$ g.	Pb $\mu$ g.	K mg.	Na mg.	Total phenols, mg.	Total enzyme mg.	Total N, mg.	Protein N, mg.
Morgan Sweet 21/6/54	4.0	7.7	16.0	1.0	10.3	0.135	74	37	7.8	6.7
Morgan Sweet 19/8/54	17.8	21.2	57.1	1.27	52.5	1.90	186	4.7	22.2	17.1
Morgan Sweet 14/10/54	25.1	33.5	81.5	8.4	86.7	4.64	236	73	34.6	25.6
Dabinett 29/6/54	2.0	5.4	9.6	0.04	9.7	0.073	84	41	8.9	6.65
Dabinett 30/8/54	7.7	16.5	36.3	2.1	43.4	0.767	192	15	22.1	14.8
Dabinett 8/11/54	12.9	29.8	92.7	6.2	69.5	3.24	237	45	26.6	19.9

iron were high in the very immature fruit and on a basis of fresh weight or dry weight fell with increasing maturity to harvest. Similar trends were observed in total and protein nitrogen. The level of sodium, however, rose with increasing maturity. In an individual fruit the amounts of all these constituents rose during growth. Polyphenol oxidase activity, however, was highest in the very immature fruit (June), dropped to very low values in August and increased again at harvest, as found by Weurman & Swain.<sup>4</sup> Between the first and second picks the amount of copper in an individual fruit increased whereas the amount of enzyme decreased. Between the second and third picks both copper and enzyme increased in an individual fruit but, when the results were expressed per unit weight of fruit, it was apparent that a decrease in copper content and an increase in enzyme activity occurred simultaneously. It is evident therefore that much of the copper present is not associated with fruit polyphenol oxidase and is likely to be available for complexing with other fruit constituents.

Freshly expressed apple juice retains some polyphenol oxidase activity which is associated chiefly with colloidal or finely dispersed tissue material since on filtration the activity is considerably reduced. Apple juice contains only small amounts of protein, and non-dialysable copper bound to protein is very small indeed. Thus, 50 ml. of freshly expressed filtered juice

originally containing 10  $\mu\text{g}$ . of copper contained after dialysis only about 0.5  $\mu\text{g}$ . of copper and 2.3 mg. of protein ( $N \times 6.25$ ). Although very little copper in apple juice is bound to protein, the presence of such complexes cannot be ignored, in view of the effect of copper in inducing sedimentation of protein-tannin-copper material in stored apple juices (Kieser *et al.*<sup>5</sup>).

Traces of copper in apple juice must therefore exist largely as cupric ions in equilibrium with copper complexed with other juice components. The nature of these complexes, the conditions under which they are formed and the amounts likely to be found in an acid medium such as fruit juice will be briefly considered.

#### (a) Organic acids

The salts of weak metallic bases and weak acids ionize abnormally in solution. Thus, cupric acetate dissolved in water gives a stable (0.01M) solution of pH 5.84 although cupric hydroxide is normally precipitated at about pH 5 (Britton<sup>6</sup>). The stability of copper salts in solution is due to association of cupric ions with the acid anions. It is convenient to include these ions or compounds formed by ionic association in the general term 'complex'. The subject of dissociation in salt solutions has been reviewed by Davies.<sup>7</sup>

The use of spectrophotometric methods, such as the method of continuous variations,<sup>8</sup> can give indications of complexes present, but where this method is inapplicable evidence for the existence of a particular complex can be obtained by exact measurement of the pH of the system. The constancy of the values found for the dissociation constant of the complex over a pH range will support the view that only this particular complex is present in solution. It is suggested on spectrophotometric grounds that the cupric salts of carboxylic acids  $\text{CuA}_2$  dissociate mainly to the first stage giving ions  $\text{A}^-$  and  $\text{CuA}^+$  (Sidgewick<sup>9</sup>). The copper complex most likely to be present in a solution of a monocarboxylic acid HA containing a cupric salt is  $\text{CuA}^+$ , especially at low pH values where the concentration of  $\text{A}^-$  is low.<sup>10</sup> In partly-neutralized dicarboxylic acid ( $\text{H}_2\text{A}$ ) solutions the copper complex most likely to be present is  $\text{CuA}$ , the formation of which is accompanied by a drop in pH, often large, caused by liberation of hydrogen ions according to the reaction  $\text{Cu}^{++} + \text{HA}^- \rightleftharpoons \text{CuA} + \text{H}^+$ . From the changes in pH, the concentrations of the various ionic species present can be determined and the dissociation constant of  $\text{CuA}$  calculated.<sup>11-13</sup>

This method was applied to systems containing malic acid, the predominant acid in apples. The dissociation constant of copper malate,  $\text{CuMal}$ , the existence of which had been demonstrated previously,<sup>12</sup> was determined and found to be relatively constant from pH 3.24 to 4.20.<sup>14</sup> The complexing of trace amounts of copper by malic acid would give rise to pH changes too small to be determined experimentally. However, equations can be derived from which it is possible to estimate the amount of free and combined copper in solutions of malic acid and copper of any known concentrations and at any pH within the range 3.24-4.20. The amounts of cupric ions, estimated from these equations, in some malic acid-copper systems at pH 3.3 in which the total copper concentration is 0.0001M (6.4 p.p.m.) are shown below (Table III).

It is seen that although  $\text{CuA}$  is almost completely dissociated at very low concentrations (0.0001M), increasing amounts of malic acid cause (as expected from the law of mass action) a reduction in its dissociation, with corresponding decrease in cupric ion concentration. Accurate values may be obtained by assessing the ionic strengths of the solutions and insertion of the appropriate activity coefficients into the equations. When this is done for a 0.065M-malate solution at pH 3.3 (comparable to apple juice) containing 0.6-6.4 p.p.m. of copper, the amount of cupric ions present is calculated to be only about 15% of the total copper.

Table III

Malic acid concentration	% Copper as $\text{Cu}^{++}$	$\text{Cu}^{++}$ , p.p.m.
M		
0.0001	98.2	6.25
0.001	87.7	4.95
0.01	34.8	2.22
0.065	7.6	0.48

Other acids such as citric acid can form copper complexes more stable than copper malate. This is readily demonstrated by the polarography of blackcurrant juice containing added cupric salt, the step height due to reduction of cupric ion being considerably reduced.

(b) *Amino-acids*

Although amino-acids can form copper complexes of high stability,<sup>15, 16</sup> the proportion of copper so complexed depends upon the pH of the solution and the concentrations of the components. This is illustrated by the work of Dobbie, Kermack & Lees<sup>17</sup> on copper-glycine complexes. The distribution of copper in the forms of cupric ions, 1:1-copper-amino-acid complexes, and 1:2-copper-amino-acid complexes of asparagine, aspartic acid and glutamic acid, the main amino-acids in apple juice, can be investigated in a similar manner. The acid pH of juice does not favour complexing, but even at pH 3.25 cupric ion concentrations are considerably reduced if the concentrations of the components are sufficiently high. Data have now been obtained for solutions at pH 3.25 containing 0.01M-amino-acid and 0.005M-cupric chloride. The percentages of cupric ions in systems containing asparagine, aspartic acid and glutamic acid were estimated as 31, 48 and 75. However, at the low concentrations of these amino-acids present in juice (0.0002-0.0010M) and with only trace amounts of copper (0.6-0.64 p.p.m.) some 85-90% of the total copper remains as cupric ions. In systems containing relatively high concentrations of amino-acids the proportion of copper fixed as complexes would be appreciable. At acid pH values, the 1:1-copper-amino-acid complexes predominate, but as the pH rises the 1:2-copper-amino-acid complexes are increasingly formed. The log  $K_s$  values (where  $K_s$  is the stability constant) of the 1:1-copper-amino-acid complexes determined at pH 3.25 were asparagine 8.14, aspartic acid 9.72, and glutamic acid 9.40. These complexes are therefore considerably more stable than copper malate (log  $K_s = 4.38$ ).

(c) *Phenolic compounds*

Copper complexes of catechol have been isolated by Weinland & Walter<sup>18</sup> and recently complex formation between copper and catechol has been followed potentiometrically.<sup>19, 20</sup> Representing catechol by  $H_2A$ ,  $CuA$  is the main complex present in weakly acid solution, while  $CuA^{2-}$  is the main complex present in alkaline solution. Again the relative proportions of free and combined copper depend upon pH and concentrations. Knowing the equilibrium constant of the reaction  $H_2A + Cu^{++} \rightleftharpoons CuA + 2H^+$  ( $pK_1 = 8.091$ )<sup>19</sup> estimations of the amounts of free and combined copper at any known concentrations of copper and catechol and at any pH below about 5 can be made from equations similar to those derived for malic acid. Calculations show that in a solution of catechol (0.01M) containing copper at a total level of 6.4 p.p.m. the amount of complexing is very small (0.04%) at pH 3.3 but that at a higher pH the complexing is appreciable (45% at pH 5.0).

The behaviour of copper in catechol systems suggests that complexing is to be expected between copper and a wide range of commonly occurring phenolics. The *o*-diphenolic grouping is present in many flavonoid and related compounds, anthocyanins and coumarins. Additional complexing of copper with flavonols can occur at the 4-keto-3-hydroxy, or at the 4-keto-5-hydroxy grouping.<sup>21-23</sup> Furthermore, the carboxyl group of the acidic phenolics may also be involved in complex formation, especially at low pH values. Current work at Long Ashton has been confined mainly to a quantitative study of the complexing of copper in systems containing the main phenolics present in apples, viz. *L-epicatechin*, *D-catechin* and chlorogenic acid.<sup>24</sup> These all contain the catechol grouping and some, such as chlorogenic acid, also contain carboxyl groups.

### Conclusions

Results so far obtained, though necessarily incomplete, now make it possible to draw some general conclusions. In plant tissues the main complexing agents are organic acids, amino-acids and related compounds<sup>25, 26</sup> and the phenolic compounds, but others such as hexitols and carbohydrate materials are not excluded. The degree of complexing depends upon relative concentrations and pH and, over pH ranges normally found in fruits, complexing can be



appreciable. Although at acid pH values the actual proportion of copper complexed with phenolic compounds may be small, this may not be unimportant as there is evidence that such complexing is the first stage in the mechanism whereby copper acts as an oxidation-reduction catalyst during oxidation of phenolic compounds.<sup>14</sup> Conversely, complex formation between copper and flavonoid and coumarin compounds in blackcurrants may be important in stabilizing the high ascorbic acid content of this fruit.

### References

- <sup>1</sup> Timberlake, C. F., *Annu. Rep. Long Ashton Res. Sta.*, 1951, p. 160
- <sup>2</sup> *Dep't. sci. ind. Res. F. Invest. Bd.*, 1953 Rep. No. 37
- <sup>3</sup> Ponting, J. D., & Joslyn, M.A., *Arch. Biochem.*, 1948, **19**, 47
- <sup>4</sup> Weurman, C., & Swain, T., *J. Sci. Fd Agric.*, 1955, **6**, 186
- <sup>5</sup> Kieser, M. E., Pollard, A., & Timberlake, C. F., *J. Sci. Fd Agric.*, 1957, **8**, 151
- <sup>6</sup> Britton, H. T. S., 'Hydrogen Ions', 1956, Vol. 2, p. 155 (London: Chapman and Hall)
- <sup>7</sup> Davies, C. W., *Endeavour*, 1945, **4**, 114
- <sup>8</sup> Job, P., *Ann. chim., Paris*, 1928, [X], **9**, 113
- <sup>9</sup> Sidgwick, N. V., 'The Chemical Elements and their Compounds', 1950, Vol. I, p. 155 (Oxford: Clarendon Press)
- <sup>10</sup> Davies, C. W., Personal communication
- <sup>11</sup> Cannan, R. K., & Kibrick, A., *J. Amer. chem. Soc.*, 1938, **60**, 2314
- <sup>12</sup> Stock, D. I., & Davies, C. W., *J. chem. Soc.*, 1949, p. 1371
- <sup>13</sup> Peacock, J. M., & James, J. C., *J. chem. Soc.*, 1951, p. 2233
- <sup>14</sup> Timberlake, C. F., *J. Sci. Fd Agric.*, 1957, **8**, 159
- <sup>15</sup> Albert, A., *Biochem. J.*, 1950, **47**, 531
- <sup>16</sup> Albert, A., *Biochem. J.*, 1952, **50**, 690
- <sup>17</sup> Dobbie, H., Kermack, W. O., & Lees, H., *Biochem. J.*, 1955, **59**, 240
- <sup>18</sup> Weinland, R., & Walter, E., *Z. anorg. Chem.*, 1923, 1923, **126**, 145
- <sup>19</sup> Näsänen, R., & Marikkanen, R., *Acta chem. fenn.*, 1956, **29**, 119
- <sup>20</sup> Timberlake, C. F., Unpublished work
- <sup>21</sup> Clarke, W. G., & Giessman, T. A., *J. Pharmacol.*, 1949, **95**, 363
- <sup>22</sup> Brune, W., *Arch. Pharm., Berl.*, 1955, **288**, 205
- <sup>23</sup> Detty, W. E., Heston, B. O., & Wender, S. H., *J. Amer. chem. Soc.*, 1955, **77**, 162
- <sup>24</sup> Williams, A. H., *Annu. Rep. Long Ashton Res. Sta.*, 1952, p. 219
- <sup>25</sup> Dobbie, H., & Kermack, W. O., *Biochem. J.*, 1955, **59**, 246, 257
- <sup>26</sup> Datta, S. P., & Rabin, B. R., *Trans. Faraday Soc.*, 1956, **52**, 1123

### Discussion

*Prof. Garner*: Is there any cuprous copper present in the complexes studied by Mr. Timberlake?

Mr. Timberlake: The complexes studied all involved cupric ions. Free cuprous ions were not very stable and split into cupric ions and metallic copper. Cuprous copper, however, can be stabilized in organic complexes, but cupric-oxygen complexes tend to be more stable than cuprous-oxygen complexes. Where phenolic compounds are concerned an equilibrium may be set up between cuprous and cupric copper, since with a reagent such as 2:2'-diquinolyl, copper is extracted as the cuprous-diquinolyl complex.

*Dr. A. Pollard* (Long Ashton): This work is complementary to that to be reported later by Dr. Mills. It shows that the behaviour, or availability, of a metal like copper may be greatly influenced by the other components present in plant tissues. The nature of these components may be as important as the level of metal present: for example, different organic acids give copper complexes that vary widely in their stability. This would also affect the nutritional properties of the plant materials.

*Dr. D. P. Cuthbertson*: Veterinary workers are interested in this subject.

*Dr. J. Tinsley* (Reading): Some workers in U.S.A. have used copper electrode systems to measure copper ion activity.

Mr. C. F. Timberlake: I have tried this but the method is a tricky one; the electrodes were easily fouled and I could not get reproducible results. The workers quoted in reference 17 in my paper had used copper electrodes.

**General discussion**

*Prof. Garner* : In catalysis those elements are most significant that have a certain structure of the internal electron shells of the atoms, especially the *d* shell. This structure is not constant for an element or a valency state, but is influenced by its combination with organic complexes. With the metallic constituents of enzymes not only the valency state but the actual nature of the combination of the metal may be important.

Element 43 has a position in the Periodic Table among the elements known to be important in catalysis ; its half-life is so short that it has disappeared during the life of the earth, but it is now being produced in the atomic piles and might be used in enzyme research.

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## THE TRACE-ELEMENT CONTENTS OF HERBAGE PLANTS WITH SOME REFERENCE TO THEIR AVAILABILITY TO THE ANIMAL\*

By A. THOMPSON

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The occurrence of unthriftiness in grazing stock resulting from sub-optimal intake of essential minerals is discussed and the practicability of supplying trace elements through the sward rather than in mineral mixtures suggested.

The trace-element contents of grass samples, artificially contaminated with known amounts of soil, are shown. The value of aluminium content as a criterion of soil contamination is considered.

Analysis of grasses, legumes and herbs indicates that the last two are, in general, better sources of the trace elements than the first; there are, however, appreciable differences within groups. Two herbs, viz., plantain and chicory, are exceptionally rich in trace elements, as in the major elements.

The effect of varying manurial treatments on the trace-element content of individual herbage species is illustrated. Certain typical moorland plants are shown to contain very large amounts of manganese, and common heather in particular to be a good source of copper and cobalt.

There is little information on the availability of trace minerals to the animal but some experimental evidence on the availability of iron in different grassland plants is presented.

Widespread disorders in livestock attributable to the deficiency or excess of a particular trace element have, in general, never been a feature of British agriculture. Because of this fact, research into the minor element contents of pastures and their component herbage plants has remained at a general interest level, and has not attained the importance which it has in countries where such disorders are both common and serious. Indeed, so rarely were deficiencies of such severity as to produce clinical symptoms diagnosed, and so local their occurrence, that the belief was widespread that most British pastures were able to meet any demands for the minor elements which might be made upon them.

In recent years, however, the emphasis upon increased production has brought into prominence the problem of sub-optimal levels of mineral intakes as against those so low as to produce clinical symptoms. Mitchell & McClure<sup>1</sup> are of the opinion that a considerable amount of unthriftiness in livestock is in no small measure due to sub-optimal intakes of the inorganic elements. The indisputable improvements in the health of stock brought about by the use of mineral mixtures is in itself some confirmation of the view that feeding stuffs, including grass and grassland products, do not always meet the demands of the animal for the inorganic elements, both major and trace.

In view of the ease with which the mineral intake of an animal may be increased by the use of mineral mixtures, the value of research into the mineral composition of pasture and herbage plants, the effect of soil and season upon that composition and the utilization of naturally occurring elements by the animal may be open to question, but this information is a pre-requisite to the intelligent use of mineral supplements. For without it and a deeper knowledge of the animal's requirements, the feeding of mineral mixtures becomes a costly routine and one not without some hazard of luxury intake.<sup>2</sup>

Although there is a considerable literature dealing with the major element contents of pasture and herbage plants, that dealing with the trace elements is by no means so extensive. In addition, much of what is available is somewhat fragmentary and the extraction of fundamental principles is made virtually impossible. Beeson<sup>3</sup> has summarized many analyses of the trace-element contents of herbage plants as well as work dealing with the effects of environmental factors upon them. Many of the species mentioned in his review, however, are not indigenous to this country, nor are soil types and climatic conditions comparable.

\* Read at Symposium on Trace Elements in Soils, Plants and Animals, organized by the Agriculture Group and Bristol Section, 10-12 April, 1957

The present contribution is, in general, based upon grassland studies in this country, and in particular upon those carried out in the Department of Agricultural Chemistry, King's College, by Brynmor Thomas, the author, and his colleagues, over the last few years. Some of this work has been fully reported elsewhere and only brief reference will be made to it here.

#### Precautions necessary in the trace-element analysis of herbage

In discussing the trace-element contents of any material, it is important to bear in mind the not inconsiderable errors associated with such determinations. Despite the many excellent methods at present available to the analyst, there must always be the possibility that contamination either before or during analysis may give rise to results which cannot be regarded with the same confidence as those for the major elements.

In this connexion it is relevant to refer to a study by Thompson & Raven<sup>4</sup> on the effects of the inclusion of extraneous soil in a sample of herbage. Particularly when such a sample is cut from a closely grazed sward, soil contamination is almost inevitable. In this investigation, grass grown by soil-less culture methods was artificially contaminated with increasing amounts of soil and then analysed for the common major and minor elements. In general, the major elements were not greatly affected by the contamination, except in so far as they showed decreases in content proportional to the amount of soil added. The results in Table I show the effect of such contamination upon the trace-element contents of the herbage.

Table I

*Trace-element composition of artificially contaminated samples of herbage (expressed on a dry-matter basis)*

Soil contamination, %	Silica (SiO <sub>2</sub> ), %	Iron (Fe), p.p.m.	Manganese (Mn), p.p.m.	Copper (Cu), p.p.m.	Cobalt (Co), p.p.m.
0	1.16	266	81	28.1	0.08
2	2.84	664	101	30.3	0.18
4	4.05	985	116	28.8	0.25
6	6.01	1381	127	28.8	0.35
8	7.88	1640	144	30.2	0.40
10	9.34	2113	155	30.1	0.45
Soil	83.60	18,740	825	35.0	4.00
Correlation coefficient	+ 0.999**	+ 0.998**	+ 0.996**	+ 0.453	+ 0.992**

\*\* Denotes significance at a probability of 0.001

The effects of the added soil upon the iron, manganese and cobalt contents are clearly shown to be of such magnitude that they cannot be ignored, whilst the copper content remains virtually unchanged. This latter effect is obviously the result of the great similarity between plant and soil copper content in this particular case, though with other soils and plants the result might have been somewhat different. A 2% soil contamination, a level which would commonly pass unnoticed, has increased the apparent iron and cobalt content of the herbage by more than 100%. It is therefore obviously important that when herbage is sampled this possible source of error is considered and every possible precaution taken to eliminate it. Thompson & Raven<sup>4</sup> are in agreement with Aston<sup>5</sup> and Shorland<sup>6</sup> that if, despite all precautions, a sample is suspected of containing extraneous mineral matter, the best estimate of contamination is obtained by determining the aluminium content of the suspected sample. Any herbage sample containing more than 150–200 p.p.m. of aluminium should be regarded as contaminated and where practicable rejected.

#### The trace-element contents of individual herbage plants

Of the many interacting factors which determine the composition of a sward in respect of a given mineral constituent, viz., the botanical composition of the sward, the chemical composition of the component herbage plants, the stages of maturity of such plants, and the soil and climatic environment, probably the most important is the difference in content between plant species. A considerable body of information exists on such inter-species differences in

the major plant constituents, but data relating to the minor elements is meagre. Beeson *et al.*<sup>7</sup> have examined 17 grasses for cobalt, manganese and copper contents, and have classified these grasses as high, medium and low, in relation to the mineral elements they supply. Unfortunately, only two of the grasses examined are indigenous to the British Isles and consequently the results have little application here.

Thomas *et al.*<sup>8</sup> have studied the mineral composition, including the trace metals, of 16 British pasture plants—8 grasses of agricultural importance, 4 legumes and 4 of the so-called herbs. These species were grown under identical soil and climatic conditions and each species was sampled at five stages of maturity. Table II shows the mean contents of the trace elements found in the individual plants as well as in the groups.

From the results in Table II it may be seen that, in general, the legumes and herbs as groups were richer sources of the trace elements than the grasses, although the differences in copper and cobalt were not great. Within the groups, however, there were some notable differences, for example, the low contents of copper, manganese and cobalt in tall fescue as compared with those of copper and manganese in cocksfoot and cobalt in red fescue. The copper and cobalt contents of tall and red fescue illustrate the fact that even species with a close botanical relationship have little in common when their mineral uptakes are compared. Two herbs, plantain and chicory, are rich sources of the trace elements as they are of the major elements, a fact which probably accounts for their reputation as valuable forage plants.

Table II

Mean trace-element contents of grasses, legumes and herbs (expressed on a dry-matter basis)

	Fe, p.p.m.	Mn, p.p.m.	Cu, p.p.m.	Co, p.p.m.
<i>Grasses</i>				
Perennial ryegrass ( <i>Lolium perenne</i> )	252	21.6	8.5	0.15
Cocksfoot ( <i>Dactylis glomerata</i> )	200	45.5	10.0	0.14
Timothy ( <i>Phleum pratense</i> )	374	30.4	7.5	0.15
Crested dogstail ( <i>Cynosurus cristatus</i> )	218	32.8	8.0	0.18
Meadow fescue ( <i>Festuca elatior</i> , sub-species <i>pratensis</i> )	250	29.2	9.5	0.16
Tall fescue ( <i>Festuca elatior</i> , sub-species <i>arundinaceae</i> )	283	23.1	4.1	0.11
Red fescue ( <i>Festuca rubra</i> var. <i>genuina</i> )	275	26.2	10.3	0.19
Chewings fescue ( <i>Festuca rubra</i> var. <i>fallax</i> )	286	17.6	6.3	0.16
<i>Legumes</i>				
Trefoil ( <i>Medicago lupulina</i> )	383	43.4	7.3	0.20
Alsike ( <i>Trifolium hybridum</i> )	323	58.8	10.5	0.17
Lucerne ( <i>Medicago sativa</i> )	291	30.9	9.1	0.15
Sainfoin ( <i>Onobrychis sativa</i> )	231	43.9	8.1	0.16
<i>Herbs</i>				
Yarrow ( <i>Achillea millefolium</i> )	294	48.3	10.6	0.17
Burnet ( <i>Poterium sanguisorba</i> )	249	31.2	8.0	0.18
Plantain ( <i>Plantago lanceolata</i> )	490	35.3	10.5	0.20
Chicory ( <i>Cichorium intybus</i> )	469	57.5	12.5	0.20
Mean of grasses	264 ± 18	28.9 ± 1.8	8.2 ± 0.7	0.15 ± 0.01
Mean of legumes	306 ± 8	45.1 ± 3.0	8.7 ± 0.5	0.17 ± 0.01
Mean of herbs	358 ± 10	41.8 ± 4.4	10.2 ± 0.7	0.19 ± 0.01

In view of the many contributory causes of variations in mineral uptake by plants, it would be unwise to suggest that a particular pasture species has anything approaching a constant mineral composition under all conditions. Nevertheless, there would appear to be some justification for the view that genetic characters play their part in determining the uptake of inorganic elements just as they determine other characteristics. For example, Mitchell<sup>9</sup> has reported the trace-element contents of ryegrass and cocksfoot and it is of interest that when these results are compared with those of Thomas *et al.*<sup>8</sup> certain points of agreement are evident for those elements common to both studies. Thus in both, cocksfoot was found to have twice the manganese content of ryegrass, though not the same absolute levels. Again, similar relative values for copper and iron contents of ryegrass and cocksfoot are reported, the latter being the higher in copper and the former in iron.

In the investigation by Thomas *et al.*<sup>8</sup> the 16 species examined at five stages of maturity showed a tendency to decline in mineral contents with advancing maturity. This decline was particularly marked in the major elements but was also in evidence with the trace elements. Thus, eight of the 16 plants gave significant negative correlations between manganese content and age, five with copper and four with iron. Even when no significant correlations were obtained, the trend suggested a declining iron, manganese and copper content with advancing maturity. With cobalt, little or no correlation was observed.

#### The trace-element contents of some hill pasture plants

In view of the somewhat more frequent occurrence of known trace-element deficiency on marginal and hill land it is of interest to consider some of the findings of Thomas & Trinder<sup>10</sup> on the ash components of some plants specific to moorland habitats. These plants were examined at six stages of maturity and large progressive differences in composition were found with advancing maturity. Table III presents mean data for these plants. The data for the composition of heather are derived from an extensive study of this plant by Thomas, Escrib & Trinder<sup>11</sup> in which heather of five different ages, namely 2, 4, 6, 8 and 10 years old, was cut four times during the season; the effects of manurial treatment upon mineral uptake by this plant were studied also.

Table III

Mean trace-element contents of some moorland plants (Thomas & Trinder<sup>10, 11</sup>)  
(expressed on a dry-matter basis)

	Fe, p.p.m.	Mn, p.p.m.	Cu, p.p.m.	Co, p.p.m.
Flying bent ( <i>Molinia caerulea</i> )	110	259	6.0	0.08
Deer hair ( <i>Scirpus caespitosus</i> )	110	399	5.8	0.08
Blaeberry ( <i>Vaccinium myrtillus</i> )	150	1835	14.4	0.10
White bent ( <i>Nardus stricta</i> )	98	469	4.9	0.10
Stool bent ( <i>Juncus squarrosus</i> )	132	192	8.1	0.08
Draw moss ( <i>Eriophorum vaginatum</i> )	132	194	8.7	0.07
Common heather ( <i>Calluna vulgaris</i> )	295	883	13.5	0.16

The results in Table III contrast markedly with those for the lowland plants considered earlier. In general, the iron contents are lower and the manganese contents very much higher in the moorland plants. Blaeberry, in particular, contained what can only be described as a phenomenal amount of manganese. The differences in copper and cobalt contents between moorland and lowland plants are not so marked. However, within the moorland group the higher copper contents of blaeberry and heather are notable, as is the cobalt content of heather. These latter data lend support to observations that when sheep have access to heather and possibly to blaeberry, copper and cobalt deficiencies are unlikely to occur.<sup>12</sup>

#### The influence of soil conditions on the trace-element composition of herbage

That variations in the mineral status of soils are reflected in changes in the composition of plants growing in them is well accepted. This interdependence is most marked with the major elements and indeed plant analysis has often been suggested as a sound method for assessing soil levels of these elements.

That swards as a whole respond to dressings of the trace elements, particularly of copper and cobalt, is so well known as to have become standard practice in areas where there are regularly such deficiencies. The precise relationships between soil and plant minor elements under more normal conditions are less well understood. It would appear to be generally accepted that uptake of these elements by the plant is governed largely by a combination of factors which include soil reaction, organic matter status, soil-water relationships, the presence or absence of antagonistic elements as well as the particular trace metal present in the soil.

In view of the fact that this whole subject is discussed by other contributors to the Symposium the topic is not further discussed, other than to refer briefly to a relevant investigation by Thomas & Thompson.<sup>13</sup> These workers have determined the mineral contents, both major

and trace, of eight herbage plants handpicked from six of the swards of the Palace Leas plots at Cockle Park, the University Experimental Station. These 16 plots have been uniformly treated with various fertilizers and manures for 50 years. Five of the six plots selected had received farmyard manure, sulphate of ammonia, basic slag, muriate of potash and a complete fertilizer respectively, while the sixth was a control untreated plot. Complete details of these manurial treatments are given elsewhere.<sup>14</sup>

Table IV shows the mean iron, manganese, copper and cobalt contents of the eight species under each treatment, and the pH values of the six plots.

**Table IV**

*Mean trace-element contents of 8 herbage plants from the Palace Leas plots (expressed on a dry-matter basis)*

Plot	Treatment	Soil pH	Fe, p.p.m.	Mn, p.p.m.	Cu, p.p.m.	Co, p.p.m.
2	Farmyard manure	5.7	118	164	12.4	0.21
6	Untreated	5.1	109	263	10.3	0.24
7	Sulphate of ammonia	4.5	137	238	9.9	0.22
8	Basic slag	5.8	101	193	9.6	0.17
9	Muriate of potash	5.0	113	368	9.6	0.21
13	Sulphate of ammonia	5.3	103	278	10.9	0.18
	Basic slag					
	Muriate of potash					

When the above results were compared with those for the major elements it was found that, in general, the trace elements were less affected by the respective treatments. The iron and manganese contents correlated with the soil reaction with only an occasional exception. This is as would be expected in view of the known relationships between plant uptake of these elements and pH.

Correlations between copper and cobalt were very much less evident. Indeed, the only copper result of note is that on the plot receiving farmyard manure which has brought about an increase in copper content of 2 p.p.m. over the control. The result is in agreement with views expressed elsewhere<sup>16</sup> that applications of organic matter increase the copper contents of plants, either by direct contribution of copper, or more probably by improving soil texture and the water relationships of the soil. Variation in cobalt contents of the herbage plants showed relatively little correlation with treatment. Mitchell<sup>9</sup> has suggested that cobalt uptake is related to the pH of the soil, amongst other factors, but the above results cannot be said to give much support to this view. It is true that the lowest cobalt contents were found on the plot having the highest pH, but it is not without significance that the treatment on this plot was basic slag. Thomas *et al.*,<sup>11</sup> working on the Tree Field plots at Cockle Park, also found a lowered cobalt content in the produce on the plot receiving this fertilizer, and yet an increase when the same rate of application was accompanied by lime.

**Variations in trace-element composition of a mixed sward**

The factors which can combine to give a sward of particular mineral composition are many. Individual plant species may differ widely in their contents of particular elements and be influenced by the stage of maturity and the particular soil in which they are growing. It therefore follows that changes in botanical composition either during any one season or in different seasons are of considerable importance. To these factors must be added those of inter- and intra-seasonal climatic differences. It is therefore not surprising to find that generalizations about the mineral contents of a sward as a whole are rarely attempted. Further study as to the relative importance of these factors is urgently needed. It could be argued that in the field the many variables will tend to compensate one another and that large variations in mineral contents may not occur. However, whilst this is a reasonable supposition, the author feels that such compensation is unlikely to completely obliterate variation. This view is supported by a study of the mineral composition of hays from the Palace Leas plots by Thomas, Holmes & Clapperton.<sup>14</sup> In this study, involving 13 of the 16 plots, only basic soil type and climate can be considered as constant for all plots. Thus, the results shown in Table V for

Table V

*Trace-element content of hays from the Palace Leas hay plots (expressed on a dry-matter basis)*

Plot No.	Manures applied*	Fe, p.p.m.	Mn, p.p.m.	Cu, p.p.m.	Co, p.p.m.	Zn, p.p.m.
1	F.Y.M., S/A, N/S, B/S, M/P	92	86	9.6	0.17	32
2	F.Y.M.	118	101	8.8	0.17	29
3	As 1, but in alternate years	125	168	9.2	0.16	36
4	F.Y.M. in alternate years	80	129	9.4	0.17	25
5	Double F.Y.M. every 4th year, other fertilizers as 1 in other years	69	169	5.4	0.16	31
6	Unmanured	83	362	6.0	0.19	37
7	S/A	240	314	6.4	0.14	37
8	B/S	186	213	9.2	0.14	45
9	M/P	109	396	7.6	0.20	43
10	S/A, B/S	94	282	5.8	0.15	40
11	S/A, M/P	92	344	6.6	0.16	31
12	B/S, M/P	60	135	7.4	0.18	31
13	B/S, M/P, S/A	84	368	8.2	0.18	35

\* F.Y.M. = farmyard manure; S/A = sulphate of ammonia; N/S = nitrate of soda; B/S = basic slag; M/P = muriate of potash

the trace-element contents of these hays may be accepted as reflecting essentially changes in botanical composition, as well as the direct effect of the fertilizers on the individual plant species.

There can be little doubt of the wide differences in trace-element composition of hays from these plots. Thomas *et al.*<sup>14</sup> conclude that the chief determining factor responsible for the above variations is change in the botanical composition of the swards occasioned by the respective manurial treatments.

#### The inclusion of herbs in pasture swards

In view of the rather remarkable mineral composition of many of the miscellaneous herbs of grassland, attempts have been made by Thomas *et al.*<sup>16</sup> to assess the effects of these species upon the yield and nutritive value of swards which contain them. It is true that the apparent superiority of these plants, as expressed by chemical analysis, lies mainly in the major elements. Nevertheless, the trace-element contents are sufficiently higher than those of the grasses for one to expect that the inclusion of herbs in seed mixtures might raise the mineral status of the sward as a whole. The idea of including such plants is not new, but there has been a revival of interest in the practice, and this has been stimulated by the observations of Stapledon<sup>17, 18</sup> and the work of Fagan & Watkins<sup>19</sup> amongst others.

In the investigation by Thomas *et al.*<sup>16</sup> plots were laid down using seed mixtures which were based on the Cockle Park mixture for a three-year ley with or without the addition of various quantities of a herb mixture. The addition of amounts of herb mixture were such as was thought would give the following establishments:

Plot	Grasses and clovers, %	Herbs, %
1	50	50
2	65	35
3	80	20
4	100	—

The herb mixture used consisted of 3 lb. of chicory, 6 lb. of plantain, 1 lb. of yarrow, 5 lb. of sainfoin and 4 lb. of burnet. In fact, in the following season, when samples were cut, a botanical analysis (on a produce weight basis) showed the percentages of the sown herbs were only 21, 14, 7 and 0%, respectively. The plots were cut 5 times during the season, the produce being weighed and dried to obtain a measure of total production during the season. The dried milled sample was then subjected to an exhaustive scheme of analysis which included some of the trace elements.

Some diminution in total yield was noted on the plots containing the greatest quantity



of herbs, but this was by no means as great as might have been expected. There was a very considerable increase in the contents of the major mineral elements, calcium being raised from 0.550% on plot 4 to 0.830% on plot 1. The results for the trace elements, although by no means so marked, nevertheless showed a useful increase in contents on the plots containing herbs.

These results are shown in Table VI.

Table VI

Mean seasonal trace-element composition of the dry matter from herb-containing swards

Plot	Fe, p.p.m.	Mn, p.p.m.	Cu, p.p.m.	Co, p.p.m.
1	296	195	10.6	2.15
2	326	216	10.7	0.16
3	288	235	8.7	0.14
4	330	225	8.1	0.09

It may be noted that the inclusion of herbs increased the level of only copper and cobalt, but these increases were found to be significant ( $p = 0.05$ ).

Further investigations into the value of the inclusion of such plants in swards are in progress at Cockle Park and the work has been extended to include studies on the possible long-term effects of such swards on animal health.

#### The availability of the element iron in herbage plants

Any discussion of the mineral contents of herbage, or indeed of any foodstuff relative to the requirements of the animal, tacitly assumes all such minerals to be completely available to the animal. While there is only meagre evidence upon which to contradict this view, evidence for its support is non-existent. However, several workers have attempted to estimate the utilization of some of the major elements, both in human foodstuffs<sup>20</sup> and recently in herbage plants.<sup>21-23</sup> In the latter studies, estimates of utilization of calcium in nine herbage plants by the rat ranged from 69.0% in *Dactylis glomerata* to 95.3% in *Plantago lanceolata*; values of the order of 60% availability for phosphorus were recorded. Evidence such as this, slender though it may be, suggests that not only is complete utilization improbable but also that herbage of various types may differ quite widely in the availability to the animal of its mineral elements. This field of research seems to be a promising one, offering the possibility of making the term 'availability' when applied to minerals as significant as that of 'digestibility' when applied to proteins.

Information as to the possible usefulness to the animal of the trace elements in grass and other herbage plants is almost entirely lacking. However, reference must be made to the work of Mills<sup>24, 25</sup> who demonstrated the existence of copper in several forms and complexes in grass, and showed that the animal could make better use of copper from herbage supplements than from an equivalent amount of cupric sulphate.

Recently Thompson & Raven<sup>26</sup> have completed an investigation into the availability of iron in eleven pasture plants, grown under identical conditions and comprising 3 grasses, 5 legumes and 3 herbs, all at the full-leaf stage of growth. While most authorities are of the opinion that iron deficiency in adult farm livestock is uncommon, the extremely small amount of this element in milk allows the possibility that in young animals anaemia caused by iron shortage may be more common than is generally accepted. Be this as it may, the present work was undertaken to see whether differences in the availability of iron as between plants were of sufficient magnitude to warrant further study, and to investigate the technique of using haemoglobin regeneration to measure the utilization of both iron and copper by the rat. Work on copper is now in progress along similar lines to that on iron, but results are not yet complete. The dangers of arguing from a small omnivore like the rat to the larger farm animals is realized, but the trials were intended as little more than bio-assays to establish whether in fact differences existed between different plant sources of iron.

The technique used was essentially that proposed by Elvehjem<sup>27</sup> and also used to considerable effect in studying human foodstuffs. The young rats were rendered anaemic, not on whole milk as suggested by Elvehjem & Kemmerer<sup>28</sup> but on the following diet :

	%
Dried skimmed milk	35
Dried whole milk	25
Sucrose A.R.	30
Vegetable fat ('Spry')	10

This diet was adopted after extensive trials which showed it to be particularly suitable. Rats grew well on it and remained in perfect health except that an acute state of anaemia could be expected within 3 weeks of commencing to feed it to weanling rats. The diet was supplemented with 0.1 mg. of copper, 0.05 mg. of manganese, yeast extract, halibut liver oil and wheat-germ oil fed daily to each rat. When the level of blood haemoglobin (Hb) dropped to 4-5 g. of Hb/100 ml., part of the basic diet was replaced by an amount of the dried, milled plant material such as would supply 0.20 mg. of iron daily.

The experiment took the form of three trials which involved the feeding of grasses, legumes and herbs respectively. In each a group of six male rats formed the control group receiving the iron supplement as ferric chloride solution, a similar number of rats being allocated to each plant iron supplement. The animals were kept in glass cages similar to the type described by Henry & Kon<sup>29</sup> with all metal parts made of pure aluminium.

The iron contents of plant species, diets and food residues were determined by the method of Parker & Griffin<sup>30</sup> and haemoglobin was estimated according to the procedure of Clegg & King.<sup>31</sup>

Table VII shows the essential data obtained from these trials. Only haemoglobin regenerations found at the end of the trial period of 28 days are shown, although intermediate determinations of blood haemoglobin were taken at 14 and 21 days. The data for mean gains in total g. of haemoglobin are based upon calculated blood volumes.

**Table VII**

*Mean haemoglobin (Hb) regeneration brought about in 28 days by various iron supplements*

Iron supplement	Fe, p.p.m.	Mean gain in Hb, g./100 ml. blood	Ferric chloride gain, %	Mean gain in total haemoglobin, g.	Ferric chloride, %
<i>Trial 1</i>					
Perennial ryegrass ( <i>Lolium perenne</i> )	242.0	0.81	46.29	0.109	50.00
Cocksfoot ( <i>Dactylis glomerata</i> )	95.3	0.93	53.14	0.105	48.17
Timothy ( <i>Phleum pratense</i> )	128.0	1.27	72.57	0.138	63.30
Ferric chloride	—	1.75	100.00	0.218	100.00
<i>Trial 2</i>					
Alsike clover ( <i>Trifolium hybridum</i> )	523.2	0.76	44.97	0.119	57.21
Broad red clover ( <i>Trifolium pratense, praecox</i> )	754.6	0.69	40.83	0.114	54.81
Kent wild white clover ( <i>Trifolium repens</i> )	718.1	0.51	30.18	0.097	46.63
Trefoil ( <i>Medicago lupulina</i> )	556.5	0.57	33.73	0.107	57.44
Lucerne ( <i>Medicago sativa</i> )	856.5	0.55	32.54	0.106	50.96
Ferric chloride	—	1.69	100.00	0.208	100.00
<i>Trial 3</i>					
Burnet ( <i>Poterium sanguisorba</i> )	299.4	0.36	20.57	0.076	38.58
Chicory ( <i>Chicorium Intybus</i> )	621.2	0.57	32.57	0.099	50.25
Plantain ( <i>Plantago lanceolata</i> )	441.7	0.68	38.86	0.108	54.82
Ferric chloride	—	1.75	100.00	0.197	100.00

The degree of closeness of the three trials in respect of response to the control ferric chloride supplements—there was in fact no significant difference between them—was very satisfactory and allows of valid comparisons between the eleven individual species.

The grasses, as a group, were significantly better sources of available iron than the herbs, the legumes occupying an intermediate position. No plant supplement was, however, as effective as ferric chloride, although timothy with an iron availability of 73% was surprisingly

good. This latter result was significantly ( $p = 0.05$ ) better than that for ryegrass and cocksfoot, between which results there was no significance.

No significance could be attached to the results for the individual legumes, with the exception of alsike and wild white clover. The iron in plantain was significantly more available than that in burnet, but even so no herb proved to be as good a source of iron as the grasses.

More detailed discussion and results will be published elsewhere, but even those presented here appear to support the view that not only is a considerable amount of the iron in plants not utilizable by highly anaemic rats which would be expected to make the best possible use of any source, but also that different plant species exhibit differences in available iron.

It is beyond the scope of this contribution to discuss the many factors which may have contributed to these results but it would not seem unreasonable to suggest that similar results will probably be found using other major and trace elements. If this proves to be so, it may occasion some change in outlook towards mineral requirement levels in animals and the probable satisfaction of these needs by herbage and feeding stuffs in general.

### References

- <sup>1</sup> Mitchell, H. H., & McClure, F. G., *Nat. Res. Coun.*, 1937, Bull. No. 99
- <sup>2</sup> Cunningham, I. J., *N.Z. J. Agric.*, 1949, **78**, 583
- <sup>3</sup> Beeson, K. C., *U.S. Dep. Agric.*, 1941, Misc. Publ. No. 369
- <sup>4</sup> Thompson, A., & Raven, A. M., *J. Sci. Fd Agric.*, 1955, **6**, 768
- <sup>5</sup> Aston, B. S., *N.Z. J. Agric.*, 1928, **36**, 22
- <sup>6</sup> Shorland, F. B., *Trans. roy. Soc. N.Z.*, 1934, **64**, 35
- <sup>7</sup> Beeson, K. C., Gray, L., & Adams, M. B., *J. Amer. Soc. Agron.*, 1947, **39**, 359
- <sup>8</sup> Thomas, Brynmor, Thompson, A., Oyenuga, V. A., & Armstrong, R. H., *Emp. J. exp. Agric.*, 1952, **20**, 10
- <sup>9</sup> Mitchell, R. L., *Research, Lond.*, 1948, **1**, 159
- <sup>10</sup> Thomas, Brynmor, & Trinder, N., *Emp. J. exp. Agric.*, 1947, **15**, 237
- <sup>11</sup> Thomas, Brynmor, Escriott, J. R., & Trinder, N., *Emp. J. exp. Agric.*, 1945, **13**, 93
- <sup>12</sup> Thomas, Brynmor, *Herb. Abstr. Rev.*, 1956, **26**, 1
- <sup>13</sup> Thomas, Brynmor, & Thompson, A., *Emp. J. exp. Agric.*, 1948, **16**, 221
- <sup>14</sup> Thomas, Brynmor, Holmes, W. B., & Clapperton, J. L., *Emp. J. exp. Agric.*, 1955, **23**, 101
- <sup>15</sup> *Bur. Soil Sci., Harpenden*, 1940, Tech. Commun. No. 39, p. 34
- <sup>16</sup> Thomas, Brynmor, Rogerson, A., & Armstrong, R. H., *J. Brit. Grassl. Soc.*, 1956, **11**, 10, 821
- <sup>17</sup> Stapledon, R. G., *J. Minist. Agric.*, 1934, **41**, 32
- <sup>18</sup> Stapledon, R. G., *Farming*, 1948, **2**, 80
- <sup>19</sup> Fagan, T. W., & Watkins, H. T., *Welsh J. Agric.*, 1932, **8**, 144
- <sup>20</sup> Kelly, J., *J. Nutr.*, 1943, **25**, 303
- <sup>21</sup> Armstrong, R. H., & Thomas, Brynmor, *J. agric. Sci.*, 1952, **42**, 454
- <sup>22</sup> Armstrong, R. H., Thomas, Brynmor, & Horner, K., *J. agric. Sci.*, 1953, **43**, 337
- <sup>23</sup> Horner, K., unpublished data
- <sup>24</sup> Mills, C. F., *Biochem. J.*, 1954, **57**, 604
- <sup>25</sup> Mills, C. F., *Brit. J. Nutr.*, 1955, **9**, 398
- <sup>26</sup> Thompson, A., & Raven, A. M., in preparation
- <sup>27</sup> Elvehjem, C. A., *J. biol. Chem.*, 1932, **98**, 1047
- <sup>28</sup> Elvehjem, C. A., & Kemmerer, A. R., *J. biol. Chem.*, 1931, **93**, 189
- <sup>29</sup> Henry, K. M., & Kon, S. K., 'Milk and Nutrition', 1937, Part 1, p. 31 (Reading: Nat. Inst. Research in Dairying)
- <sup>30</sup> Parker, W. E., & Griffin, F. P., *Canad. J. Res.*, 1939, **17(B)**, 66
- <sup>31</sup> Clegg, J. W., & King, E. J., *Brit. med. J.*, 1942, ii, 329

### Discussion

*Mr. S. K. Cowlshaw* (Grassland Research Institute): As an animal agronomist I am particularly interested in the work on the availability of trace elements. The data presented on the total content of trace elements in individual herbage species and in different families do not always correspond with the findings of Dr. Mitchell. Dr. Mitchell found less manganese in red clover than in two grasses. How much soil contamination was associated with the data in Table II? What was the magnitude of the variability between replicate analyses of single herbage samples? To what extent does this variability affect the validity of comparisons between the trace-element content of individual herbage species?

Mr. Brynmor Thomas (on behalf of the author): The soil contamination has been watched carefully and we have taken due care to use plated mills and nickel chrome scissors. All the analyses were replicated and the differences obtained between the groups were highly significant.

*Dr. F. E. Moon* (Fisons Ltd.): Has any examination been made of the trace-element content of different strains of the same herbage species? There is some New Zealand work indicating that there may be quite big differences in composition between strains.

Mr. Brynmor Thomas : Some work has been attempted on strain differences without success. A limited amount of work on the major elements has been done by Fagan, but the only substantial differences obtained are in respect of calcium in lucerne.

*Mr. A. M. Frens* (Haorn, Holland) : The differences in manganese content noticed by an earlier speaker in the work by Dr. Mitchell and Mr. Thompson could be explained by pH difference in the soil. It is well known that the uptake of manganese by plants is strongly related to soil pH. On acid soils the manganese content of herbage is much higher.

Mr. Thomas : pH does make an enormous difference to the manganese content. These experiments were carried out on a soil of high pH and also on Palace Leys.

*Dr. G. E. G. Mattingley* (Rothamsted) : Has Mr. Thomas any information on the trace-element content of the fertilizers used at Cockle Park and are the amounts removed annually by crops comparable with the amounts added as fertilizer impurities ?

Mr. Thomas : The only data available are those for the actual soils.

*Dr. R. L. Mitchell* (Macaulay Institute) : In our experience, on soils high in cobalt, clovers contain more cobalt than grasses, while the grasses, particularly cocksfoot, are higher in manganese. Normally, lower iron contents than those reported by Thompson are found, although there are occasionally similar levels. Differences in soil availability, possibly related to organic matter, have to be kept in mind. Normally a reduction in cobalt and manganese is found after liming, particularly of cobalt in clover and of manganese in grasses. There is little effect on iron in our soils.

Mr. Thomas : Differences in respect of cobalt content are not significant, and in general our results agree with those of Dr. Mitchell. No relationship between soil pH and cobalt uptake can be established. Plot 8 of Old Tree Field had lime in addition to slag, and the grass had a higher cobalt content than that on Plots 3 and 4 which had received slag only.

*Mr. E. A. N. Greenwood* (Sutton Bonington) : The work at Sutton Bonington can throw light on the discrepancy of the manganese figures quoted in the discussion. Manganese experiments with lucerne, repeated at different times of the year, gave variable results. When the plants were moved into conditions of controlled light and temperature it was found that the level of illumination in particular had a profound effect on the manganese content.

## FACTORS INFLUENCING THE BLOOD-COPPER LEVEL OF SHEEP: THE EFFECT OF CHANGE IN BASAL METABOLIC ACTIVITY\*

By HAMISH A. ROBERTSON and ARTHUR W. J. BROOME

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The effect of change in basal metabolic activity as induced by the administration of thyroxine and thiouracil has been studied in relation to the level of blood copper in sheep. Elevations in blood-copper values of up to 75% have been observed after thyroxine administration, the values remaining high for up to 50 days. Depressions of almost 50% in blood-copper values have been observed after thiouracil administration, these values returning fairly rapidly to normal on cessation of treatment. No change in blood-copper values was observed during administration of DNOC for 40 days.

### Introduction

It has become generally recognized that the assessment of the copper status of a flock of sheep by determining the blood-copper levels of a random sample may not always be reliable. In any such determination a very wide scatter of values may be found, which when plotted do not always give a random distribution curve. It would therefore seem appropriate to study in the sheep those factors that are known to influence the level of copper in the blood of animals. By such means it may be possible to explain the abnormal values which arise and also to obtain some information relating to more fundamental problems of copper metabolism.

No conditions associated with hypocupraemia apart from those of dietary origin have been described in domestic animals, and only in the disorders of nephrosis and hepato-lenticular degeneration in the human has hypocupraemia of non-dietary origin been described. Hypercupraemia occurs more frequently, and in man a rise in plasma-copper has been observed in most acute and chronic infection. Robertson<sup>1</sup> has shown that sharp rises in blood-copper levels can occur in sheep as a consequence of acute infections such as tick-borne fever and that the high level may persist for as long as six weeks after the termination of the febrile phase. In spite of copious literature on the subject, the correlation between the changes in the levels of blood copper and the infection is not really known. During the febrile phase of the infection there is an increase in general cellular metabolism, and as a consequence of this and of the haemo-concentration which occurs, an increase in blood-copper value might be expected. It is difficult, however, to account for the very considerable increases (up to 100%) which occur.

Under normal conditions the thyroid gland plays a considerable part in the chemical regulation of body temperature by virtue of the effects of its secretions on cellular metabolism. Different authors have demonstrated in animals<sup>2</sup> and in man<sup>3, 4</sup> that there is a correlation between thyroid activity and blood-copper values. It seemed therefore of interest to study the effect of changes in basal metabolic activity induced by thyroxine, thiouracil and dinitro-ortho-cresol (DNOC) on the blood-copper level of sheep.

### Experimental

#### *Animals*

Two-year-old Clun Forest ewes were kept indoors and fed a constant diet for six weeks before commencing the experimental period. Samples of blood were taken regularly during this preliminary period. No control animals were kept, the response to treatment being assessed by comparing the blood levels of the animal during the preliminary and experimental periods.

#### *Administration of thyroxine*

Sodium-L-thyroxine was dissolved in sterile dilute NaOH solution. The volume was then made up to 5 ml. with water and given immediately by intramuscular injection. A review of the literature gives very little indication of the amount of sodium-L-thyroxine likely to produce

\* Read at Symposium on Trace Elements in Soils, Plants and Animals, organized by the Agriculture Group and Bristol Section, 10-12 April, 1957

a suitable metabolic response. Blaxter<sup>5</sup> related metabolic activity to dose of iodinated casein when given daily by mouth but it is difficult to evaluate the relative potency of the iodinated casein used in his experiments in terms of pure sodium-L-thyroxine.

#### Administration of thiouracil

Since thiouracil was given in fairly large quantities it was administered by mixing the desired quantity with 50 ml. of water and drenching the sheep with the mixture. The literature is inconclusive as to the dose of thiouracil required to inhibit thyroid activity in the sheep. The initial dose used was based on the values of Barrick, Andrews & Beeson<sup>6</sup> and the precaution was taken of measuring blood-cholesterol as an indication of thyroid activity.

#### Administration of DNOC

The DNOC was dissolved in alkali, made up to 5 ml. and given by intramuscular injection. The toxic dose of DNOC, like that of dinitrophenol, is very difficult to assess. It can build up in the blood to high levels without apparently affecting the animal and then quite suddenly it reaches a threshold value and produces serious effects, this threshold value being markedly influenced by environmental temperature and humidity. According to Kamel<sup>7</sup> the mean LD<sub>50</sub> for mice is 25 mg./kg. In view of this it was decided to proceed cautiously by giving gradually increasing doses.

#### Methods of analysis

(1) *Copper*.—For the copper determination the wet digestion method of Eden & Green,<sup>8</sup> using 2-ml. quantities of oxalated whole blood, was employed. The diethyldithiocarbamate complex was determined spectrophotometrically at 430 m $\mu$  with a Unicam S.P. 500 instrument.

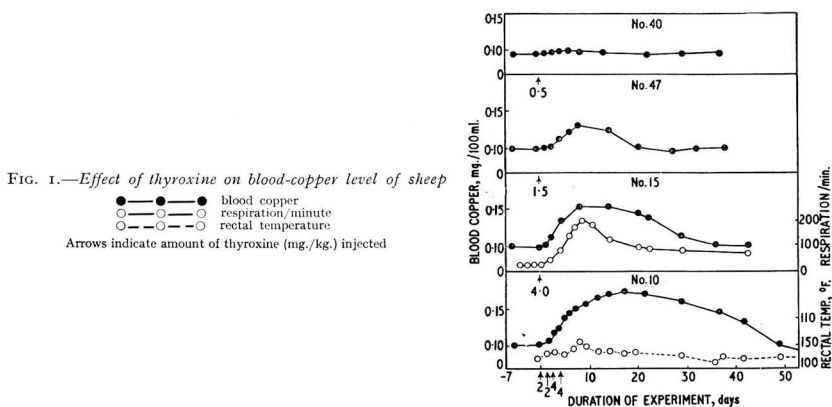
(2) *Cholesterol*.—Plasma-cholesterol determinations were carried out on 1-ml. samples extracted with 5 ml. of boiling acetone-ethanol (2 : 1 v/v) for 2 min. The extracts were cooled, made up to 10 ml. and filtered. Two-ml. portions of the filtrate were taken for estimating total cholesterol by the digitonin procedure, modified by Sperry & Webb.<sup>9</sup>

## Results

### Effect of thyroxine

Fig. 1 demonstrates the effect of increasing amounts of thyroxine on the concentration of copper in the whole blood of four sheep.

With sheep No. 40 weighing 80 kg. no physiological symptoms nor any effect on blood-copper level following a single injection of 0.5 mg. of thyroxine/kg. were evident. With sheep



No. 47 weighing 56 kg., however, a definite rise in blood-copper level was observed following the administration of 1.5 mg. of thyroxine/kg. This change was not associated with any observable change in respiratory rate or rectal temperature.

In an attempt to produce some definite physiological derangement of the normal metabolic activity, a large single dose of 4.0 mg. of thyroxine/kg. was administered to sheep No. 15 weighing 80 kg. On the third day the animal began to exhibit slight symptoms of hyperthyroidism. These increased in severity until on the 11th day the respiratory rate had risen from a pre-treatment level of 40/min. to 240/min. with the animal showing marked signs of distress. The respiratory rate was still high (70/min.) 50 days after the single injection. In this animal the blood-copper level rose from a pre-treatment level of 0.096 mg./100 ml. to a maximum around 0.150 mg./100 ml. on the 11th day and did not return to pre-treatment levels until 40 days after treatment. During the 50-day period after the thyroxine administration the animal lost 11 kg. in weight.

In order to ascertain just how high the blood-copper levels might rise sheep No. 10, weighing 75 kg., was given a total of 900 mg. of thyroxine over a period of four days. Symptoms of hyperthyroidism did not become apparent until the day after the final injection had been given. At this time the respiratory rate and the rectal temperature of the animal were high but there was little else to suggest that the animal was abnormal. During the next day the symptoms of hyperthyroidism progressively increased, the animal became nervous and lost its appetite. This state continued for five weeks before the animal gradually regained its appetite and appeared normal. At the conclusion of the experiment, 60 days after the commencement, this animal had lost 14 kg. in weight.

It is of interest to note that although the rectal temperature rose from around 102.5° F to its maximum value of 105.5° F on the tenth day after commencement of dosing and was back to a normal pre-treatment level on the 30th day, the blood-copper level did not attain its maximum until about the 17th day and did not return to normal until the 49th day after treatment, i.e., approximately 20 days after the return to normal of body temperature.

*Effect of thiouracil*

It is extremely difficult to assess the degree of hypothyroidism and the depression of basal metabolic activity during a short-term treatment with thiouracil. Since change in plasma-cholesterol level is a fairly sensitive guide to changes in thyroid activity, plasma-cholesterol levels were followed in order to be quite certain that a state of hypothyroidism had been attained.

The results of dosing sheep No. 1 weighing 85 kg. with thiouracil is illustrated in Fig. 2. It would appear that at the 2.5 mg./kg./day level little change in either blood-copper or plasma-cholesterol values took place. On progressively increasing the dose of thiouracil, rapid changes

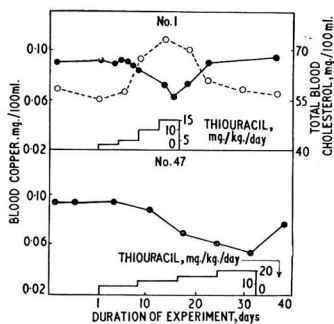


FIG. 2.—Effect of thiouracil on blood copper of sheep

in both these values became apparent, the blood copper falling from an initial value of 0.920 mg./100 ml. to 0.620 mg./100 ml. with a daily dose of 15 mg. of thiouracil/kg. The total plasma-cholesterol value rose from an initial value of 58.0 to 73.0 mg./100 ml. Both of these values returned to normal fairly rapidly when thiouracil treatment ceased.

Sheep No. 47 weighing 56 kg. and having previously been used to demonstrate the effect of thyroxine on blood-copper levels (Fig. 1) was given a more prolonged course of treatment with thiouracil. The results obtained are similar to those for sheep No. 1.

#### Effect of DNOC

Fig. 3 illustrates the effect of gradually increasing doses of DNOC on sheep No. 35 weighing 87 kg. Over a period of 35 days a total of 1.90 g. of DNOC was given intramuscularly in

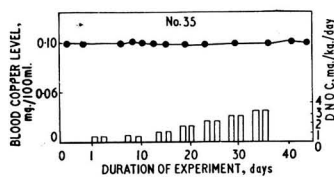


FIG. 3.—Effect of DNOC on blood-copper level of sheep

gradually increasing doses. It is evident that no change in blood-copper concentration was produced by this level of dosing, neither were there any physiological symptoms suggestive of increased metabolic activity. The rectal temperature measurements were all normal, *viz.* between 102.3 and 103.3° F.

A single dose of 15 mg./kg. administered to sheep No. 18 weighing 75 kg. produced no harmful effect on the animal and there was no change in blood-copper level.

#### Discussion

It is apparent from the above results that the activity of the thyroid gland must be an important factor in influencing the blood-copper concentration of sheep. An elevation of 75% and a depression of 50% of the value of the blood copper have been produced by increasing and decreasing the level of circulating thyroid hormone.

The duration of response appears to be much longer with thyroxine than with thiouracil. This might suggest that under field conditions the possibility of obtaining a misleading value of blood-copper level following a short period of change in thyroid activity would be greater in the case of hyperactivity when high values would be obtained.

The manner in which the thyroid produces these changes in blood-copper level is a matter for considerable speculation. Although it might be possible to attribute some of these changes to changes in blood volume following thyroxine and thiouracil treatment, this explanation cannot account for the large changes demonstrated. In considering the possible origin of the relatively considerable quantities of copper mobilized following thyroxine treatment the various possibilities are either mobilization from the liver or increased absorption, since decreased excretion during a period of general increase in cellular activity would be unlikely. Calculations show that the liver would normally contain ample copper to cover the increase in blood level. It is doubtful, however, if it would be possible to demonstrate the mobilization of this amount of copper from the liver even by using a biopsy technique. With regard to the depression of blood-copper values following thiouracil treatment Kozelka & Pedrero<sup>10</sup> reported that thyroidectomy of rats does not affect liver or kidney copper values.

It has not been possible to demonstrate whether the effects produced on the blood-copper level are the result of changes in thyroid hormone level *per se* either directly or through some thyroid-pituitary-adrenal interrelationship, or whether these changes are associated with general changes in cellular activity. It was hoped that part of this question would have been resolved



by the DNOC treatment. In the two experiments carried out, however, the results of the DNOC treatment are inconclusive inasmuch as although there was no change in blood-copper level at the dose levels used, it would clearly be necessary to demonstrate changes in cellular metabolism as a consequence of DNOC treatment before any conclusion in this respect can be drawn.

#### References

- <sup>1</sup> Robertson, H. A., Ph.D. Thesis, University of Edinburgh, 1950
- <sup>2</sup> Naraska, S., *Japan J. med. Sci., II, Biochem.*, 1938, **4**, 25
- <sup>3</sup> Locke, A., Moin, E. R., & Rosbach, D. O., *J. clin. Invest.*, 1932, **11**, 527
- <sup>4</sup> Nielsen, A. L., *Acta med. scand.*, 1944, **118**, 431
- <sup>5</sup> Blaxter, K. L., *J. agric. Sci.*, 1950, **9**, 389
- <sup>6</sup> Barrick, E. R., Andrews, F. N., & Beeson, W. M., *J. Anim. Sci.*, 1950, **9**, 389
- <sup>7</sup> Kamel, S. H., *Brit. vet. J.*, 1955, **111**, 34
- <sup>8</sup> Eden, A., & Green, H., *Biochem. J.*, 1940, **34**, 1202
- <sup>9</sup> Sperry, W. M., & Webb, M., *J. biol. Chem.*, 1950, **187**, 97
- <sup>10</sup> Kozelka, F. L., & Pedrero, E., *Fed. Proc.*, 1952, **11**, 364

#### Discussion

*Dr. E. J. Butler (Moreduin)*: Some years ago I found that thiouracil forms stable complexes with copper. Can this property depress the blood-copper level? Can Dr. Robertson give more information on his work relating to the influence of infection and pregnancy on the blood-copper level of sheep?

*Dr. Robertson*: Thiouracil has a number of different effects in the animal and these all require consideration. Thiouracil has a generalized pharmacological effect on normal cellular metabolism in addition to its more specific action in blocking the iodination processes in the thyroid. It should be remembered that in addition to a lowering of the general metabolic activity as a consequence of the lowered levels of circulating thyroid hormone a shift takes place in the relative rates of secretion of certain anterior pituitary hormones, viz. thyrotrophin increases while there is a decrease in the gonadotrophic hormones, F.S.H. and I.C.S.H.

With due regard to these considerations it is thought that it is the lowering of metabolic activity which is probably of importance in the decrease in blood-copper level and not the effect due to the production of a copper chelate with thiouracil. Besides, the thyronines, including thyroxine, also form chelates with copper.

Changes in blood-copper level have been studied in cases of tick-borne fever, louping ill and enzootic abortion. It would appear that the greatest changes are associated with febrile conditions.

It has been reported that in humans an increase in blood-copper level occurs during pregnancy, while in the sheep under field conditions this has not been demonstrated. If experiments on the sheep were carried out under conditions of controlled environment differences might be observed. It is of interest to note that in the human, increases in the level of protein-bound iodine have been reported during pregnancy. This does not appear to occur in sheep although it is thought that no sufficiently critical work for, or against, any change in protein-bound iodine levels during pregnancy has yet been published.

*Dr. E. C. Owen*: Some years ago, in work with thiouracil and thyroxine, I used for determination their effect on milk alkaline phosphatase. A cow injected with 20 mg. of thiouracil showed an increase in milk phosphatase comparable with the decrease caused by 5 mg. of L-thyroxine. With regard to the effect of these two drugs on blood-copper I would like to point out that there is an analogy with the effect of thyroxine and vitamin A assimilation from the gut.

*Dr. Robertson*: I agree that variations in the rate of absorption is a factor to be considered. An increase in gut motility occurs with increase in blood level of thyroid hormone and the converse also applies. Even under normal conditions it is very difficult to carry out a satisfactory

copper-balance experiment, which in any case yields very little information since absorbed copper can be excreted back into the intestinal tract and appear as unabsorbed copper in the faeces.

*Dr. R. Allcroft*: In a study of the protein-bound iodine levels in blood during pregnancy, we found no rise in copper measured on whole blood but perhaps there might be in plasma.

*Dr. P. L. van Erkelens*: In the work of Dr. P. C. Hart reported in the *Netherlands Agric. J.*, it is shown that methyl-thiouracil does not affect the copper level of the liver.

*Dr. Robertson*: Technically it might be very difficult to demonstrate changes in liver-copper associated with changes in blood level even if the former occurred. A complicating factor would be the changes in liver weight which would occur.

*Dr. F. E. Moon*: What influence has adrenaline on blood-copper level? Violent exercise increases blood-copper levels in sheep, whereas mild exercise does not, and this suggests that fright and consequent adrenaline secretion may be a common factor in one case but not in the other.

*Dr. Robertson*: Because of vaso-constriction and decrease in blood volume an increase in copper concentration as part of general increase in concentration of blood constituents might occur. However, the problem is much more complex. Adrenaline may exert a direct effect on the thyroid increasing the rate of thyroid hormone secretion. In addition consideration must be given to the effect of adrenaline directly or indirectly on the adrenal-cortex since the corticosteroids have an effect on the thyroid.

## DIETARY FACTORS INFLUENCING COPPER UTILIZATION BY THE ANIMAL\*

By C. F. MILLS

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Previous work on dietary factors which may affect the utilization of copper in the animal is reviewed. Studies on the stability and solubility of organic complexes of copper in herbage and the utilization of these compounds by the rat and sheep are described and the results discussed in relation to the suggestion that certain stable, soluble complexes may be directly absorbed through the digestive tract mucosa.

### Introduction

The effect of inorganic dietary factors on the copper metabolism of the animal has recently been extensively studied. Much of this work has been reviewed by Underwood,<sup>1</sup> and other aspects, particularly copper/molybdenum/sulphate relationships, will be considered by other contributors to this Symposium.

Although it has been shown that the elements zinc<sup>2, 3</sup> and molybdenum<sup>4, 5</sup> markedly interfere with copper metabolism when present in high levels in the diet, little is known of their mode of action. Furthermore it is apparent that neither of these elements can be incriminated as the sole cause of many conditioned copper-deficiency disorders of cattle and sheep in Britain<sup>5, 6</sup> where the copper content of available fodder<sup>7</sup> is well above levels normally recognized as adequate by Australasian workers.<sup>8, 9</sup> It appears possible that at least three distinct processes could result in copper deficiency of livestock under these conditions:

- (1) a dietary factor or metabolic defect resulting in increased output of copper in the urine,
- (2) failure to utilize copper absorbed into body tissues,
- (3) failure to absorb copper from the digestive tract.

### *Factors affecting urinary copper output*

Although Wilson's disease in man is associated with excessive urinary excretion of copper, mainly in the form of specific Cu-oligopeptide complexes,<sup>10</sup> no such metabolic disorder has yet been found in the ruminant. Attempts to increase urinary copper excretion in the sheep by injection of B.A.L. have so far proved largely unsuccessful<sup>11, 12</sup> although this effect is readily obtained in man.<sup>13</sup> It has been suggested that molybdenum, in the presence of dietary sulphate, may increase urinary copper output in the sheep but direct experimental evidence is as yet lacking.<sup>14, 15</sup>

### *Factors believed to restrict physiological utilization of stored copper*

It has been clearly demonstrated with sheep,<sup>16, 17, 18</sup> rats and swine<sup>19</sup> that dietary molybdenum and sulphate may induce a 'physiological' deficiency of copper before tissue copper levels have become markedly depleted. Recent studies of zinc toxicity in the rat indicate that this element may similarly impede the utilization of stored copper since the activity of the liver cytochrome oxidase system—a sensitive index of copper deficiency—is markedly depressed by high levels of zinc but returns to normal when the copper intake is increased.<sup>3</sup> The feeding of an excessive quantity of zinc does not appear to influence the extent of copper storage by the liver.<sup>19</sup>

### *The effect of other factors on copper uptake and storage*

As has recently been stressed, the factors affecting the absorption of copper from the digestive tract are imperfectly understood and have so far received little attention.<sup>1</sup> This is undoubtedly due to the difficulty of conducting satisfactory balance trials in which contamination of urine and faeces can be rigidly excluded. In most studies, changes in the copper content of liver or blood are taken as the criteria of altered absorption—measures which unfortunately

\* Read at Symposium on Trace Elements in Soils, Plants and Animals, organized by the Agriculture Group and Bristol Section, 10-12 April, 1957

fail to eliminate effects due to altered rates of copper excretion in urine or bile and which fail to account for possible changes in the 'physiologically available' copper level of body tissues.

Thus choline deficiency has been found to reduce the storage of copper in the liver<sup>20</sup> and the inclusion of excessive choline in the diet increases the toxicity of high levels of copper to the rat.<sup>21</sup> Hypocupraemia has been reported in swine during protein deficiency<sup>22</sup> and pyridoxine deficiency.<sup>23</sup> It has been suggested on indirect evidence<sup>23, 24</sup> that pantothenic acid may be concerned in the utilization of dietary copper in the rat but we have been unable to confirm this suggestion in recent studies.<sup>25</sup>

#### *Copper utilization in relation to the nature of ingested copper*

The effect of copper administered in the form of a wide variety of different compounds has been studied by a number of workers. Thus copper in the form of copper 'caseinate', glycineamide biuret, alanineamide biuret, citrate, aspartate, glutamate, nucleinate, oxide, hydroxide and pyrophosphate, and copper in haemocyanin was found to be readily utilized by the rat<sup>26</sup> whereas the copper of copper haematoporphyrin and copper sulphide was not absorbed. It has been claimed that copper phyllocyanate [a complex isolated from green plants<sup>27</sup> to which has been ascribed the formula  $(C_{24}H_{27}N_2O_4)_2Cu$ ] is not utilized by the rabbit<sup>28</sup> despite earlier reports to the contrary.<sup>27</sup>

These findings stimulated our interest in the nature of copper compounds present in food materials. It was felt that before the complexities of conditioned copper-deficiency disorders of ruminants could be fully understood, information would be required on the solubility and stability of copper compounds in the ingesta and the changes undergone by these materials in the digestive tract.

Preliminary studies<sup>29</sup> *in vitro* indicated that the greater part of the copper in herbage remained insoluble after treatment with a wide range of aqueous and organic solvent systems. Examination of soluble fractions by dialysis against dilute solutions of chelating agents and by filter-paper chromatography indicated that most of the soluble copper was present as organic complexes, the copper being bound in a form more stable than the cupri-glycinate complex. Investigations on the stability of these complexes in aqueous digests, their availability to the rat, and studies of their fate in the digestive tract will now be reported.

### **Experimental**

The techniques used in preparing black-hooded rats for copper availability studies have been described previously.<sup>30</sup> Response to supplementation of a deficient diet with copper in different forms is measured by growth rate, by haemoglobin recovery in the anaemic animal, by the rate of recovery of melanin pigmentation in hair and by copper analysis of the liver. In initial experiments the utilization of copper given as  $CuSO_4 \cdot 5H_2O$  was compared with that obtained from the feeding of equivalent quantities of this element in samples of freeze-dried herbage. The responses obtained with cupric sulphate were in all measures inferior to those obtained by the inclusion of herbage supplements in the diet. Later work<sup>30</sup> indicated that the response obtained with herbage was associated with components present in an aqueous extract.

Since it had been shown that most of the copper in such a fraction was present as organic complexes it was suggested that copper in the form of certain of these materials may readily be utilized by the animal. Before this postulate can be accepted it is necessary to demonstrate (i) that the aqueous fraction of herbage does not liberate appreciable amounts of the free cupric ion under the conditions of acidity likely to be met in the digestive tract, (ii) that herbage extracts from which traces of ionic copper and complexes of low stability have been removed retain their physiological activity, and (iii) by direct chemical methods, if possible, that such complexes exist in the digestive tract.

#### *Gastric acidity in the black-hooded rat*

The pH of the stomach contents of copper-deficient rats 8–10 weeks of age was determined within 1 minute of death by insertion of a glass/calomel electrode assembly through an incision

in the stomach wall. Results are presented in Table I. These results indicate that pH fluctuations within the range 2.8–4.3 might be expected at different times after ingestion of the diet used in copper availability trials.

**Table I**

*pH of stomach contents of rats taken within 1 minute of death*

Time after feeding Cu-deficient diet (h.)	2	6	10	17
Range of pH values	4.3–4.0	3.8–3.5	3.8–2.8	3.6–3.4
Mean	4.1	3.7	3.3	3.5
(No. of observations)	(4)	(4)	(4)	(3)

*The effect of cation-exchange resins on in vitro acid digests of the soluble components of herbage*

A 1:10 suspension of ground freeze-dried herbage in glass-distilled water was continuously stirred at 37° for 18 hours, filtered through muslin and centrifuged at 8300g for 1 hour. Five-ml. samples of supernatant were incubated for 3 hours with 0.005 g. of pepsin after adjustment of the pH to required values with 2N-HCl. After cooling, 2 g. of Zeokarb-215 (Na<sup>+</sup>) was added to each digest and the pH readjusted to its original value with acid. After stirring gently for 1 hour the supernatant was decanted, the resin washed with 3 × 5 ml. of glass-distilled water and the supernatant and resin washings pooled for copper analysis. Copper uptake by the resin was determined from the difference in copper content of the digest before and after resin treatment (Table IIa). Above pH 2 copper uptake by the resin was small, less than

**Table II**

(a) *Copper uptake by Zeokarb-215 (Na<sup>+</sup>) from in vitro digests of aqueous extracts of herbage*

Cu content of untreated extract: 1.77 µg.

pH of digest	Residual Cu after resin treatment (µg.) (Mean of 3 determinations)	% of total Cu retained by resin
4.0	1.60	9.6
3.0	1.65	6.8
2.5	1.60	9.6
2.0	1.55	12.4
1.5	1.00	43.5
0.9	0.55	68.9
0.5	0.60	66.1
0.3	0.90	49.1

(b) *Copper uptake by Zeokarb-215 (Na<sup>+</sup>) from Na solution of the electrolysable cations of herbage + added cupric ions*

Cu content of untreated extract: 1.96 µg.

pH of solution	% of total Cu <sup>++</sup> retained by resin
4.0	94.9
2.8	92.3
2.0	92.3
1.3	89.8
0.9	87.2
0.5	82.1
0.3	64.3

10% of the total being retained. Since this fraction remained constant over the pH range 2–4 it is probably largely due to the adsorption of copper-containing materials on the resin matrix rather than to uptake of free ions from solution. Between pH 1 and pH 2 a greater uptake of copper was observed, presumably due to the decomposition of complexes in the digest solution. Below pH 1 copper retention by the resin becomes less efficient owing to cation competition for the exchange sites of the matrix, as was shown by following the uptake of inorganic Cu<sup>++</sup> ions added to a solution containing the freely ionizable cations of a herbage extract which had been separated by electrolysis (Table IIb).

*The physiological utilization of copper from herbage extracts treated with a cation-exchange resin*

It had been found earlier that columns of Zeokarb-215 charged with a variety of cations will readily decompose the relatively stable cupri-glycinate complex, it being possible to retain copper ions on the resin while obtaining complete elution of free glycine. The effect of resin pre-treatment on the physiological utilization of copper from herbage extracts was then investigated. Full details of experimental techniques have been previously published.<sup>31</sup> The physiological response of the copper-deficient rat to the feeding of 3  $\mu\text{g}$ . Cu/day in a Zeokarb-215-treated aqueous herbage extract was compared with that obtained from different levels of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  administered *per os*. Results are summarized in Table III.

**Table III**

*Group mean values and standard errors for haemoglobin (Hb) level, weight gain, liver Cu and change in hair pigmentation index\* of Cu-deficient rats fed Cu supplements for 30 days*

Treatment	Hb level g./100 ml. blood		Weight gain, g.	Total liver Cu, $\mu\text{g}$ .	Change in pigmenta- tion index	No. of animals
	Initial	Final				
1. 3 $\mu\text{g}$ . $\text{Cu}^{++}$ /day	6.1	9.4 $\pm$ 0.6	19 $\pm$ 4	9.7 $\pm$ 0.8	+ 1	6
2. 6 $\mu\text{g}$ . $\text{Cu}^{++}$ /day	5.6	9.2 $\pm$ 0.6	25 $\pm$ 4	10.9 $\pm$ 0.8	+ 1	6
3. 10 $\mu\text{g}$ . $\text{Cu}^{++}$ /day	5.9	11.8 $\pm$ 0.6	30 $\pm$ 4	12.8 $\pm$ 0.9	+ 2	5
4. 15 $\mu\text{g}$ . $\text{Cu}^{++}$ /day	5.9	11.8 $\pm$ 0.5	35 $\pm$ 4	13.0 $\pm$ 0.7	+ 2	8
5. 3 $\mu\text{g}$ . copper/day in resin- treated herbage extract (see text)	6.1	11.1 $\pm$ 0.6	30 $\pm$ 4	13.2 $\pm$ 0.9	+ 2	6

\* Based on an arbitrary pigmentation scale from 10 (indicating a fully pigmented black-hooded rat of normal Cu status) to 1 (indicating severe depigmentation of hair in acute Cu deficiency)

Significant differences were obtained in the rate of haemoglobin recovery during the 30-day experimental period ( $P < 0.001$ ) with treatments 1 and 2 (see Table III) giving poorer recovery than treatments 3, 4 and 5. This pattern of treatment effects was reflected in differences in weight gain, hair pigment regeneration and liver copper levels at the end of the experiment although these differences just failed to reach the 5% level of significance. These results indicate that a herbage extract from which  $\text{Cu}^{++}$  ions and cationic copper complexes have been removed by resin treatment still contains copper in a form which can be assimilated by the rat.

*Studies of the fate of  $^{64}\text{Cu}^{++}$  in the digestive tract of the sheep*

*Electrodialysis of digestive tract fluids.*—Irradiated 'Specpure' Cu foil (0.04 g. with specific activity approx. 50 mc./g.) was dissolved in conc.  $\text{HNO}_3$  and excess acid neutralized with 2N-NaOH. This preparation was mixed with 50 g. of freeze-dried herbage and 20 g. of sucrose and fed to a 70-lb. sheep. The animal was killed 19 hours later and samples of rumen, abomasum and duodenal liquor were centrifuged at 8000g for 1 hour at  $-5^\circ$ . Fifty-ml. samples of each supernatant were electro dialysed for 9 hours in four-compartment electro dialysis units<sup>32</sup> using toughened filter paper and Cellophane membranes. The  $^{64}\text{Cu}$  activity of dialysis cell contents was determined by liquid counting (Table IV).

Electrodialysis of an inorganic system containing added  $^{64}\text{Cu}^{++}$  ions gives 95–97% recovery of added radioactivity in the cathode compartment after a 6-hour dialysis period. Although trace levels of activity were detected in the catholyte obtained from electro dialysis of rumen and abomasum fluids, the counts obtained were not significantly above background. That free cations migrated is clearly shown by the changes in pH of the catholytes during electro dialysis. In all cases a small proportion of the  $^{64}\text{Cu}$  migrated from the sample compartment through the filter-paper membrane in the direction of the cathode but was denied access to the cathode compartment by the Cellophane membrane. Since the  $\text{Cu}^{++}$  ion readily penetrates this membrane the results suggest that the free  $\text{Cu}^{++}$  ion concentration of the original preparations was small.

*Chromatographic studies of digestive tract contents of the sheep.*—The free  $\text{Cu}^{++}$  ion may be separated from many stable organic complexes by filter paper chromatography.<sup>29, 33</sup> Attempts are being made to use this procedure to study the nature of copper compounds in digestive tract fluids.

Table IV

*Electrodialysis of digestive tract fluids from a sheep after radioactive  $^{64}\text{Cu}^{++}$  administration per os*

	Membrane	pH of cell contents after electro-dialysis	$^{64}\text{Cu}$ in cell after 9-h. electro-dialysis Decay and background corrected, count/min.	Equivalent Cu content, $\mu\text{g}$ .
A. Rumen liquor				
1. Anode cell		< 2	5	*
2. Sample cell	Cellophane	6.1	1972	7.12
3. " "	Filter paper	6.9	50	0.18
4. Cathode cell	Cellophane	> 10	6	*
B. Abomasum liquor				
1. Anode cell		< 2	10	0.04
2. Sample cell	Cellophane	3.9	215	0.78
3. " "	Filter paper	5.0	47	0.16
4. Cathode cell	Cellophane	> 10	3	*
C. Duodenal liquor				
1. Anode cell		> 2	0	*
2. Sample cell	Cellophane	6.8	12186	43.9
3. " "	Filter paper	7.2	48	0.17
4. Cathode cell	Cellophane	> 10	2	*

\* Count not significantly above background

Radioactive  $^{64}\text{Cu}$  was administered to sheep as outlined above and samples of fluid later withdrawn from the rumen, abomasum and duodenum. After centrifuging at 30,000g for 30 minutes at  $-1^{\circ}$ , 0.012-ml. samples of supernatant were applied to Whatman 3 MM paper and chromatograms developed for 18 hours with phenol/water as (descending) solvent. After drying the papers autoradiographs were obtained on Ilford 'Industrial G' X-ray film. Results are presented in Table V. The technique as described has two important limitations—(i) the active spots obtained in the  $R_F$  range 0.0-0.05 appear to be due to the denaturation of radioactive material on the paper during the drying process: this is particularly unfortunate since it is in this area that the free  $\text{Cu}^{++}$  ion would lie if present in these extracts; (ii) the sensitivity of the autoradiographic method is slight and the quantity of  $^{64}\text{Cu}$  (half-life 12.8 hours) cannot be increased if unphysiological levels are to be avoided. It is hoped to overcome these difficulties by an improved preparative procedure and by employing radiometric scanning rather than autoradiography, but the results obtained so far clearly demonstrate the existence of organic complexes of copper in the digestive tract fluids. The identity and origin of the highly active spot of  $R_F$  0.65-0.75 in the duodenal fluid are in doubt. It was thought that it might have originated in the bile but chromatographic examination of uncontaminated bile has so far failed to confirm this.

Table V

*Filter paper chromatography of  $^{64}\text{Cu}$  complexes in the digestive tract fluids of the sheep*

	Solvent system: phenol/water (descending) $T = 20^{\circ}$					
	$R_F$ of segments showing autoradiograph response					
	0.0-0.05	0.10-0.15	0.20-0.25	0.50-0.55	0.65-0.75	0.85-0.90
Rumen liquor	+++	+	+	+	—	+
Abomasum liquor	+++	+	+	—	—	—
Duodenal liquor	++	—	+	—	++	—

+ Indicates positive response on Ilford 'Industrial G' X-ray film

— Indicates no response

## Discussion

Studies in which aqueous extracts of herbage were incubated *in vitro* at different pH values with pepsin indicate that within the range of acidity likely to be found in the gastric contents of the rat the greater part of the soluble copper remained 'bound' in the form of neutral or anionic complexes. Extracts treated with a sulphonic acid exchange resin to remove any free  $\text{Cu}^{++}$  ion and cationic complexes still contained copper in a form which could be assimilated by the rat, comparative results indicating that the copper of such extracts was rather more readily utilized than copper given *per os* as cupric sulphate. It has been suggested<sup>34</sup> that effects similar to this obtained in earlier work<sup>30</sup> may result from the use of the sulphate salt of copper in view of recent observations on the importance of sulphate-molybdenum inter-relationships in copper metabolism. Although no figures are available for the total sulphate content of the basal diet used in the work reported above it seems likely that the additional contribution made by the cupric sulphate supplements would be insignificant.

Studies involving electro dialysis and chromatography demonstrated the existence of 'bound' forms of soluble copper in digestive tract fluids of the sheep and no significant liberation of the free  $\text{Cu}^{++}$  ion could be detected even in the most acid regions of the tract.

These observations lead to the suggestion that the transport of cupric ions through the intestinal mucosa may not be the only mechanism whereby this element is absorbed—it appears possible that certain soluble complexes of copper present in food materials may also be absorbed intact. Such a concept of copper transport through a biological membrane in the form of intact complexes is not new and its possibility has been indicated by the results of other workers also.<sup>10, 13, 35-37</sup>

The fact that inorganic copper administered to a copper-deficient animal results in copper uptake and storage in no way precludes absorption of this element as complexes since, as has been shown, such copper readily combines with materials present in the digestive tract. Since many of the products of the reaction of copper with biological materials are insoluble, however, the overall utilization of copper in this form is poor.<sup>38, 39</sup>

The aim of work now in progress is to study, using these techniques, the effects of feeding to the ruminant diets known to cause conditioned copper-deficiency disorders. By so doing it is hoped to indicate whether these disorders are the result of abnormal copper metabolism within the digestive tract or, alternatively, the end effects of a failure of copper metabolism during or after the absorption of this element through the digestive tract mucosa.

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## References

- Underwood, E. J., 'Trace Elements in Human and Animal Nutrition', 1956, p. 59 (New York: Academic Press)
- Smith, S. E., & Larson, E. J., *J. biol. Chem.*, 1946, **163**, 29
- Van Reen, R., *Arch. Biochem. Biophys.*, 1953, **46**, 337
- Dick, A. T., *Soil Sci.*, 1956, **81**, 229
- Allcroft, R., & Lewis G., *Landbouwk. Tijdschr.*, 's Grav., 1956, **68**, 711
- Stewart, J., Farmer, V. C., & Mitchell, R. L., *Nature, Lond.*, 1946, **157**, 442
- Innes, J. R. M., & Shearer, G. D., *J. comp. Path.*, 1940, **52**, 249
- Beck, A. B., *J. Dep. Agric. W. Aust.*, 1941, **18**, 285
- Cunningham, I. J., Paper in 'Copper Metabolism—a Symposium on Animal, Plant & Soil Relationships', eds. McElroy, W. D., & Glass, B., 1950, p. 246 (Baltimore: Johns Hopkins Press)
- Uzman, L. L., *Amer. J. med. Sci.*, 1953, **226**, 645
- Green, H. H., Paper in 'Plant and Animal Nutrition in Relation to Soil and Climatic Factors', 1951, p. 293 (London: H.M.S.O.)
- Stewart, J., Paper in 'Plant and Animal Nutrition in Relation to Soil and Climatic Factors', 1951, p. 281 (London: H.M.S.O.)
- Denny-Brown, D., & Porter, H., *New Engl. J. Med.*, 1951, **245**, 917
- Dick, A. T., *Aust. J. agric. Res.*, 1954, **5**, 511
- Dick, A. T., Personal communication



## References (contd.)

- <sup>16</sup> Dick, A. T., Paper in 'Inorganic Nitrogen Metabolism', eds. McElroy, W. D., & Glass, B., 1956, p. 445 (Baltimore: Johns Hopkins Press)
- <sup>17</sup> Marston, H. R., *Physiol. Rev.*, 1952, **32**, 66
- <sup>18</sup> Wynne, K. N., & McClymont, G. L., *Aust. J. agric. Res.*, 1955, **7**, 45
- <sup>19</sup> Kulwich, R., Hansard, S. L., Comar, C. L., & Davis, G. K., *Proc. Soc. exp. Biol. Med.*, 1953, **84**, 487
- <sup>20</sup> Hegsted, D. M., Kinney, T. D., & Cartaya, J. A., *Amer. J. Path.*, 1948, **24**, 722
- <sup>21</sup> Kaufman, N., Cartaya, J. A., White, P. L., Hegsted, D. M., & Kinney, T. D., *J. Nutr.*, 1952, **46**, 433
- <sup>22</sup> Cartwright, G. E., & Wintrobe, M. M., *J. biol. Chem.*, 1948, (a) **176**, 571; (b) **172**, 557
- <sup>23</sup> Singer, L., & Davis, G. K., *Science*, 1950, **111**, 472
- <sup>24</sup> Hundley, J. M., & Ing, R. B., *Fed. Proc.*, 1951, **10**, 385
- <sup>25</sup> Mills, C. F., & Murray, G., Unpublished results
- <sup>26</sup> Schultze, M. O., Elvehjem, C. A., & Hart, E. B., *J. biol. Chem.*, 1934, **106**, 735; 1936, **115**, 453
- <sup>27</sup> Tschirch, A., 'Das Kupfer', 1893 (Stuttgart: Ferd. Enke)
- <sup>28</sup> Taylor, A. E., Long, J. H., & Chittenden, R. H., U.S. Dep. Agric., 1913, Rep. 97
- <sup>29</sup> Mills, C. F., *Biochem. J.*, 1954, **57**, 603
- <sup>30</sup> Mills, C. F., *Brit. J. Nutr.*, 1955, **9**, 398
- <sup>31</sup> Mills, C. F., *Biochem. J.*, 1956, **63**, 190
- <sup>32</sup> Syngé, R. L. M., *Biochem. J.*, 1951, **49**, 642
- <sup>33</sup> Mills, C. F., & Murray, G., *Nature, Lond.*, 1956, **177**, 793
- <sup>34</sup> *Nutr. Rev.*, 1956, **14**, 317
- <sup>35</sup> Saha, K. C., & Bannerjee, S., *Ann. Biochem.*, 1943, **3**, 131
- <sup>36</sup> Seelemann, M., & van Bandissin, F., *Zbl. Veterinarmed.*, 1954, **1**, 354
- <sup>37</sup> Havinga, A., & Bykerk, R., *Rec. Trav. chim. Pays-Bas*, 1947, **66**, 184
- <sup>38</sup> Comar, C. L., Davis, G. K., & Singer, L., *J. biol. Chem.*, 1948, **174**, 995
- <sup>39</sup> Schubert, G. W., Maurer, W., & Riezler, W., *Z. ges. inn. Med.*, 1948, **3**, 170

## Discussion

*Dr. E. J. Butler*: Has osteoporosis been observed in the copper-deficient rats at the Rowett Institute? It is interesting to see that this is a primary feature of the copper deficiency produced experimentally in puppies and pigs by Baxter and his co-workers in U.S.A.

*Dr. Cuthbertson* (on behalf of the author): I am under the impression that we have not observed this. [Subsequent discussion with Dr. Mills, however, has indicated that he has observed brittleness of bone in all cases of severe copper deficiency.]

*Prof. Wallace*: In studies of the copper status of pastures in swayback areas, indicator plants were used on arable plots for treatments with and without copper. Addition of copper had no beneficial effect on plant growth and was sometimes toxic: nevertheless, the sheep on the surrounding untreated pasture suffered from swayback.

*Miss G. Lewis* (Weybridge): Sheep taken from swayback farms in Derbyshire were fed hay from their home farms, containing 9 p.p.m. of copper. Other sheep were fed on a diet of oats containing excess of copper, cobalt and sulphur. The sheep on the supplemented food had a significantly higher liver copper and no swayback.

*Dr. Robertson*: The report in the paper that BAL has no effect on urinary excretion of copper in sheep is based on a misconception. In field trials, 10 sheep injected with a low level of BAL over 14 days showed no change in blood-copper level, but large intramuscular injections of BAL did increase the urinary output of copper.

*Dr. E. C. Owen*: In the ruminant, with extra thyroxine, calcium is found in the faeces and not in the urine. In pregnancy the animals are unlikely to excrete either calcium or copper.

*Mr. H. S. Foster*: Is the anomalous result obtained at Weybridge with sheep brought from Derbyshire and fed on home farm hay, due to the fact that they were fed hay? It is known that hay from teart pastures does not produce copper-deficiency symptoms, though the fresh grass does.

*Dr. R. Allcroft*: In some cases hay has produced swayback.

*Dr. Nicholas* : An excess of zinc has been found to reduce the activity of cytochrome-*c*-oxidase in rat liver ; this could be restored by adding copper to the diet. Like zinc and copper, molybdenum and copper have been found reciprocally antagonistic, and in micro-organisms this effect can be explained in terms of enzymic changes which appear worth studying in connexion with animals.

*Mr. W. S. Ferguson* (Jealotts Hill) : Did the examination of the copper constituents of swayback pasture reveal any differences compared with a pasture which did not cause swayback ?

*Dr. Cuthbertson* : There was a small difference in the amount of copper complexes but their nature was unknown.

*Mr. P. J. Warren* (Bristol) : Has Dr. Mills found any evidence of demyelination in copper-deficient rats ?

*Dr. Cuthbertson* : No.

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## COPPER NUTRITION IN RUMINANTS. DISORDERS ASSOCIATED WITH COPPER-MOLYBDENUM- SULPHATE CONTENT OF FEEDING STUFFS\*

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### Introduction

The first suggestion that deficiency of copper occurs naturally in grazing animals was made in the early 1930's by Neal *et al.*<sup>1</sup> in America and by Sjollega<sup>2</sup> in Holland. Both these reports referred to the condition in cattle. They were followed by the important work of Bennetts & Chapman<sup>3</sup> who showed that enzoötic ataxia, a demyelinating disease of lambs which occurred on copper-deficient pastures in Western Australia, was a manifestation of copper deficiency, which was preventable by administration of copper to the ewes during pregnancy, or by increasing the copper content of pastures by fertilizing the soil with copper salts. This disease occurs in sheep in other parts of Australia, in New Zealand, in South Africa, and in many parts of Great Britain, where it is known as 'swayback'. Innes & Shearer<sup>4</sup> showed that swayback corresponds pathologically to the enzoötic ataxia of Australasia but they and other British workers have shown that it differs aetiologically in that its occurrence is not associated with a simple copper deficiency of the herbage.

Bennetts & Hall<sup>5</sup> and Bennetts<sup>6</sup> in Western Australia also reported another enzoötic disorder, 'falling disease' in dairy cattle, characterized by loss of condition, sudden death and low copper status. This disease was also associated with a very low copper content of pastures and was preventable by administration of copper to the cattle or by top dressing the pastures with copper salts.

Later reports by Cunningham<sup>7, 8</sup> have described two copper-deficiency disorders of cattle in New Zealand, one associated with a simple deficiency of copper in pastures and the other 'a complicated copper deficiency' associated with pastures moderately low in copper and high in molybdenum.

Copper deficiency in cattle in Britain was first confirmed in 1946<sup>9</sup> and has subsequently been observed in many parts of the country.<sup>10</sup> As in the copper-deficiency syndrome in sheep in Britain, hypocuprosis in cattle occurs on pastures of apparently normal or even high copper content. Reports from Holland<sup>11</sup> and Eire<sup>12</sup> show a similar relationship between copper deficiency in cattle and apparent copper sufficiency in pastures. This indicates the presence of other factors in the food which interfere with utilization or storage of copper in the animal.

In this paper it is intended to refer briefly to some of the investigational and experimental work carried out at Weybridge on copper nutrition in cattle and sheep and to relate the results to those reported by other workers in this field. Since the clinical and pathological manifestations in the two types of animal are so dissimilar and since there appear to be differences in their copper metabolism, it is convenient to discuss them separately.

### Hypocuprosis in cattle

A wide variety of clinical symptoms have been associated with copper deficiency in cattle, but none of them are specific. The chief ones are progressive loss of condition and a harsh, dull, discoloured coat; diarrhoea is not always present. In Britain, concurrent anaemia is uncommon but it has been reported in Western Australia<sup>6</sup> and in New Zealand.<sup>8</sup> Adult as well as growing cattle are affected but young stock usually show most severe symptoms and frequently (especially in beef herds) the calves are affected while their mothers appear clinically normal. In severely affected animals the response to administration of small amounts of copper is usually spectacular. Nothing resembling the neo-natal demyelinating disease of lambs has been reported in calves in Britain.

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Hypocuprosis has been observed in many areas throughout the country and our evidence does not indicate that it is confined to any particular soil type. It is usually found in varying degrees of severity in cattle on 'teart' (Lower Lias clay) and peaty areas, but is by no means confined to them.

*Effect of molybdenum.*—In 1943 Ferguson, Lewis & Watson<sup>13</sup> showed that the scouring disease of cattle known locally as 'teart' which occurred on the Lower Lias clay outcrops in Somerset, Gloucester and Warwick, was due to a high molybdenum content of soil and pasture.

'Teart' mainly affects cows in milk and young stock, and diarrhoea may commence within two days of putting cattle on to affected pastures in spring; the animals develop staring, discoloured coats and lose condition rapidly, and the milk yields drop. Ferguson *et al.*<sup>13</sup> showed that the diarrhoea could be controlled and animals maintained in good health on 'teart' pasture by the oral administration of copper sulphate. They did not, at that time, investigate the mechanism of the scouring effect of the high molybdenum intake or its control by copper sulphate, nor was the copper status of cattle in the 'teart' area examined.

In 1945 Dick & Bull<sup>14</sup> in Australia showed that a high molybdenum intake reduced liver copper storage in cattle and sheep and thus brought copper-molybdenum relationships into prominence. Cunningham<sup>8</sup> confirmed and amplified this work and showed that a scouring disease of cattle on peat land in New Zealand was associated with a primary deficiency of copper upon which was superimposed a moderate excess of molybdenum.

Other scouring diseases have also been associated with a high molybdenum content of soils and pastures in various parts of the world, on Californian clay loam,<sup>15</sup> Florida peat soils,<sup>16</sup> Irish peats and loams of limestone derivation,<sup>17</sup> and in parts of the Swan River valley in Manitoba.<sup>18</sup>

Russell<sup>19</sup> put forward the view that 'teart' was a conditioned copper deficiency. At Weybridge the scouring effect of 'teart' pastures has been regarded as a straightforward molybdenosis<sup>20, 21</sup> since it could be produced in a time which seemed too short to allow depletion of the animal's copper status to deficiency levels. Support for this view was given by experimental observations<sup>22</sup> on stalled cattle given sodium molybdate orally and intravenously to produce scouring. Liver biopsy samples showed a decrease in liver-copper concentration after about a week, but when scouring and loss of condition occurred 12–20 days after commencement of molybdenum administration, liver copper values were still more than 40 p.p.m. and within a range considered normal for cattle. Continued administration of molybdenum for two to three weeks after onset of scouring did not produce a further appreciable fall in liver copper.

It appears, therefore, that scouring and loss of condition in cattle can occur as a result of molybdenosis without a concomitant hypocuprosis. If, however, the high intake of molybdenum is continued over several months, liver-copper storage will be reduced ultimately to deficiency levels of below 15 p.p.m. (dry wt.). At this stage the scouring effect of molybdenum may become more persistent and severe as it appears to do in 'teart' areas and in some peat areas.

Since no observations on the copper status of animals in the 'teart' area of Somerset were reported by Ferguson *et al.*,<sup>13</sup> investigations were carried out at Weybridge.<sup>21</sup> Blood samples from cattle on 'teart' farms which had not had prophylactic copper supplements showed low copper values; heifers of normal copper status sent from Weybridge to a 'teart' farm commenced scouring in one to two weeks and blood copper fell from normal to low levels after two to three months.

Diarrhoea in cattle on 'teart' farms and in experimental animals at the laboratory on a continued high molybdenum intake was controlled for periods of three to five weeks by a single intravenous injection of a suitable amount of copper sulphate solution. This form of treatment was found as effective as oral supplements of copper sulphate and has subsequently been used extensively for treatment of copper deficiency in beef herds and in other cattle for which oral supplementation has not been convenient. It should be emphasized, however, that intravenous copper treatment can have harmful effects if too much or too strong a solution of copper sulphate is given.

Experimental observations on stalled and grazing animals at this laboratory agree with

Cunningham's findings<sup>8</sup> that an increased intake of molybdenum reduces liver-copper storage, produces scouring and unthriftiness and reduces growth rate in cattle. Field evidence,<sup>21-24</sup> however, does not suggest that an abnormal intake of molybdenum is the only factor concerned in producing a low copper status in bovines with resulting loss of condition with or without concomitant diarrhoea. This aspect will be discussed in more detail in the section dealing with the copper-molybdenum-sulphate contents of pastures.

### Hypocuprosis in sheep

The chief manifestation of copper deficiency in sheep in Britain is the occurrence of 'swayback' or neo-natal ataxia in lambs. The essential symptom is a spastic paralysis which varies in severity, while the pathological characterization is a diffuse symmetrical demyelination of the cerebrum with secondary degeneration of the motor tracts of the cord. The disorder is always associated with low blood- and liver-copper values in 'swayback' lambs and their mothers. Many liver samples, however, from ewes and lambs have shown equally low copper values without any evidence being found of demyelination or of clinical manifestations of swayback in the flock, but there is ample proof that administration of copper to the ewe during the gestation period will prevent the occurrence of swayback in lambs.

No anaemia or other abnormality of the blood picture has been observed in sheep with a low copper status, as has been reported from Australia.<sup>25</sup> The clinical condition appears to be unaffected; no 'pining' symptoms such as may occur in copper-deficient cattle have been noted in sheep, nor have the abnormalities of the fleece described by Lee<sup>26</sup> ('the loss of normal crimp, the appearance of secondary waves and the production of typically straight and unusually lustrous fibres') been observed. This may, however, be due to lack of detailed observation and the fact that most of our studies on copper deficiency in sheep have been made on the small mountain breeds which have long, straight, hair-like wool with no natural crimp. But in one copper-deficient flock of Swaledale ewes in Cumberland in which swayback did *not* occur, the fleeces were harsh and bleached white. The same effect on the fleece has been noted in some groups of ewes in which copper deficiency has been induced experimentally at the Laboratory. This depigmentation and harshness of the wool are similar to the changes in physical characteristics which usually occur in the hair of copper-deficient cattle and small laboratory animals.

The occurrence of copper deficiency in sheep in Britain on pastures of normal copper content suggests the presence of factors interfering with the storage and utilization of copper. Field and experimental evidence has shown no relationship between copper and molybdenum content of feeding stuffs and incidence of swayback.<sup>21, 27</sup> Later work<sup>28</sup> indicated that the nature of the diet rather than the total copper and/or molybdenum content influenced liver-copper storage in the ewe and foetus.

About this time, the outstanding work of Dick<sup>29-33</sup> in Australia demonstrated the significance of the inorganic sulphate content of the diet on the inhibiting effect of molybdenum on liver-copper storage in stalled sheep.

Dick<sup>30</sup> suggested that some of the discrepancies in the reports of workers in Australasia and Great Britain might be due to the different intakes of inorganic sulphate by the animals under the conditions recorded. The authors<sup>34</sup> therefore examined the inorganic sulphate, copper and molybdenum contents of diets in relation to liver-copper storage in ewes and new-born lambs, and re-investigated the effect of increasing the molybdenum intake of both pregnant and barren ewes on different types of diet which supplied basic copper and molybdenum intakes within the normal range. For these experiments no suitable diet could be found which would supply a 'low normal' sulphate intake of less than 0.5 g. per day as postulated by Dick<sup>32</sup> (all cereal straws and hays examined to date have shown sulphate contents far higher than the oat chaff which constituted Dick's low-sulphate diet). Therefore, all the diets used contained ample sulphate to allow the molybdenum present to exert its full limiting effect.

Under these conditions it was found that increasing the inorganic sulphate 3-5 times had no significant effect on liver-copper levels in pregnant ewes of low or normal copper status. The results for lambs, however, suggest that, when the maternal copper status is low, foetal liver copper can be reduced by a high sulphate intake, and that kale, either because of its

high sulphate content or presence of other constituents, may exert a significant inhibiting effect.

When the molybdenum intake was increased 50 times and 14–200 times the basal intake for periods of 5 and 15 months respectively on diets of constant and ample sulphate content, the results indicated that the limiting effect of molybdenum was influenced by various factors such as the initial copper status of the ewes, the copper content of the diet and foetal requirements.

These experiments were conducted over a period of four years, using new batches of sheep of various breeds for each experiment which extended over the gestation period. The different results produced by variations in animals and diets confirmed Dick's<sup>30</sup> suggestion that previous discrepancies between Weybridge and Australasian reports could be accounted for by differences in the conditions under which the trials were carried out.

#### Pasture data

As previously stated, the chief aetiological difference between copper-deficiency syndromes in sheep and cattle in Australasia and in Great Britain is that in the former they are associated with pasture copper values which are regarded as deficient, while in the latter they are associated with pasture values regarded as containing adequate or even high concentrations. Bennetts<sup>6</sup> reports that results of surveys of pastures in Western Australia by Beck indicate that, where the copper content of pasture is above 6 p.p.m., stock remain healthy, that with values between 3 and 6 p.p.m. signs of copper deficiency may be expected, and that severe clinical manifestations occur on pastures with values below 3 p.p.m. Marston<sup>25</sup> reports that on and near the southern Australian littoral the indigenous pastures rarely contain more than 4 p.p.m. and frequently much less. The copper reserves of mature sheep grazed there become depleted and copper-deficiency symptoms appear. In New Zealand Cunningham<sup>8</sup> regards the mean copper content of 'normal' pastures to be 11 p.p.m. and has found that 'simple copper deficiency' occurs in cattle and sheep maintained on pastures with a mean copper content of 3 p.p.m. It is only rarely that pastures with mean copper values below 5 p.p.m. are found in Britain. Results<sup>34</sup> given in Table I show that there is no significant difference in copper content of pastures on which deficiency symptoms do or do not occur in cattle and sheep.

Cunningham's reports on 'peat scours'<sup>8, 35</sup> indicate that the pathological effect of molybdenum on cattle is determined by the relative amounts of copper and molybdenum in the pastures. A moderate excess of molybdenum causes the disorder only if the pasture copper is below normal. For example, if the pasture contains about 10 p.p.m. copper, about 20 p.p.m. molybdenum must be present before toxic effects are produced; for lower levels of copper the minimum harmful level of molybdenum is lower, so that molybdenum is harmful at levels of 3 to 7 p.p.m. when pasture contains only 3 to 5 p.p.m. copper.

The results in Table I suggest that this relationship does not hold in Britain. On pastures with mean copper and molybdenum values of 24 and 7 p.p.m. respectively, severe copper deficiency has been found. Earlier data<sup>21</sup> indicated that high pasture molybdenum was not a contributing factor in the occurrence of swayback and the figures in Table I support that observation.

Dick's experimental work<sup>32</sup> demonstrated the vitally significant effect of the sulphate content of the diet on the limiting effect of molybdenum. The inorganic sulphate figures in Table I indicate that ample amounts are present to allow the molybdenum to exert its full limiting effect. This suggests that a difference in sulphate content is not a factor contributing to the occurrence or absence of copper deficiency in animals grazing these pastures. Cunningham<sup>36</sup> has also reported that there appears to be sufficient sulphate in New Zealand pastures from areas where copper deficiency occurs with and without molybdenum excess to enable the molybdenum present to exert its full effect on copper elimination.

Both experimental and field evidence so far obtained at Weybridge indicate that the copper, molybdenum and inorganic sulphate contents of the food are not the only factors concerned in the occurrence of hypocuprosis in cattle and sheep. They also suggest that a low copper status of ewe and lamb does not invariably lead to swayback. It seems that another factor is concerned which operates only when copper deficiency is also present.

**Table I**

*Copper, molybdenum and inorganic sulphate content of pastures on dry matter basis*

Description	No. of samples	Copper		Molybdenum		Inorganic sulphate	
		p.p.m.		p.p.m.		g.-%	
		Mean	Range	Mean	Range	Mean	Range
<i>Sheep pastures</i>							
1. 'Non-Swayback'							
(a) Somerset	10	10	8.5-12	19	5.7-26	0.61	0.41-0.96
(b) Other areas	44	18	6.2-3.5	1.2	0.39-2.5	0.61	0.23-0.91
2. 'Swayback'	15	14	8.4-20	1.6	0.46-3.6	0.68	0.45-0.95
<i>Cattle pastures</i>							
1. No copper deficiency							
(a) Non-industrial	20	12	6.2-21	1.1	0.32-2.1	0.67	0.49-0.90
(b) Industrial	11	22	1.5-3.5	3.5	2.0-7.5	0.83	0.40-1.3
2. Copper deficiency							
(a) Non-industrial	5	15	1.3-1.8	2.1	1.1-4.3	0.72	0.44-0.82
(b) Industrial	13	24	1.3-3.7	7.0	1.5-15	0.58	0.24-1.1
(c) Somerset	15	10	7.5-13	12	3.6-26	0.59	0.30-0.96

Since there appear to be several dietary factors which can influence assimilation and utilization of copper, it is difficult to assess the minimum copper requirements of cattle and sheep. Even from this brief review of the problem it is evident that the copper concentration of food-stuffs may be of limited value in estimating a 'normal' intake of copper for the animal. Underwood<sup>37</sup> has pointed out that a 'true' or basic minimum copper requirement can be conceived as one in which all the dietary conditions affecting copper are at an optimum and that such an optimum cannot yet be given. Dick<sup>38</sup> has suggested that crossbred stalled sheep can, under appropriate dietary conditions, be in copper balance on an intake of about 1 mg. per day or less, which is very much lower than has generally been believed possible. His data also indicated that the amount of copper required to replace normal wastage was less than 3 mg. per day and he suggests that where more than this is required by sheep to maintain their copper status, other factors are operating which either impose a limitation on copper assimilation and retention or increase the animal's requirement for copper.

The question of the chemical forms in which copper occurs in herbage and its availability to animals is being studied by Mills.<sup>39-41</sup> He has shown<sup>40</sup> that a greater physiological response and storage of liver copper in the rat resulted from the feeding of a stable water-soluble copper complex from both 'normal' and 'swayback' herbage than from the corresponding quantity of copper administered as cupric sulphate. It may be of interest to mention that a recent trial, although not comparable with Mills' work<sup>40</sup> because the hay itself and not a water-soluble copper extract was used, in which one group of pregnant ewes were fed hay from a farm with a long history of 'swayback' and other groups were fed different diets supplemented with copper, molybdenum and sulphate to give similar intakes, indicated that copper given orally as copper sulphate was much more readily available to both ewe and foetus than similar amounts of copper present in the 'swayback' hay.

**Chronic copper poisoning**

Because of the widespread use of copperized mineral supplements for farm stock in this country and because chronic copper poisoning appears to have become more prevalent over the last few years, a discussion of factors which can cause excessive copper storage would be relevant in this review.

When small amounts of copper are continuously ingested in excess of the quantity required for physiological equilibrium, the tissues accumulate abnormal amounts, especially the liver which acts as the main storage depot and provides the most reliable index of the copper status of the animal. Under some circumstances, and depending on the nature of the diet, especially in sheep and cattle, liver-copper storage increases considerably, the amount accumulated varying with the species and the individual. Until certain high levels are reached, there appears to be no unfavourable effect on the animal, but further accumulation may lead to a sudden

liberation of copper into the blood stream resulting in extensive haemolysis, jaundice and death.

Before Boughton & Hardy<sup>42</sup> described chronic copper poisoning in sheep due to ingestion of excessive amounts of a copperized salt lick, the condition had been reported only infrequently and incompletely. The most comprehensive investigation of this condition has been carried out by Australian workers<sup>25, 43, 44</sup> in connexion with the occurrence of chronic copper poisoning in grazing sheep in Australia. Bull<sup>44</sup> has described its occurrence there under three different field conditions: (1) when the copper contents of soil and herbage are high, (2) when both these are within the normal range, and (3) in association with liver damage due to poisoning by the plant *Heliotropium europaeum*, abundant growth of which occurs in summer pastures on certain areas under favourable weather conditions. Under (1) the condition appears to be caused by a straightforward high intake of copper due to the high copper content of the pastures and by their contamination with cupriferous soil. Some plant species in these areas have shown copper contents up to 50–60 p.p.m. There is no seasonal incidence, and deaths from chronic copper poisoning occur in every month of the year. The condition under (2) is usually seasonal in occurrence and the incidence is much higher in British breeds or crosses than in Merinos and is associated with a dominant pasture growth of subterranean clover (*Trifolium subterraneum*) which commonly contains 10–15 p.p.m. of copper but very low levels of molybdenum, usually less than 0.1 p.p.m. The evidence suggests that the actual intake of copper and the copper/molybdenum ratio are important factors in the development of the disease. That other factors are also involved is strongly supported by the pen feeding experiments of Dick<sup>32, 38</sup> which have demonstrated the significance of the inorganic sulphate content of the diet. The disease associated with the consumption of *H. europaeum*, i.e., condition (3), has been shown<sup>45</sup> to be essentially due to the altered metabolism of the liver cell resulting in an atrophic hepatitis. Excessive amounts of copper are stored—usually 1000–2000 p.p.m. or more—and the liver damage predisposes sheep to the haemolytic crisis of chronic copper poisoning.

Bull<sup>44</sup> has reported that there appears to be no malnutrition associated with any of these types and the characteristics are a more or less sudden haemaglobinaemia and haemaglobinuria, usually with icterus. Necrosis of the liver, kidney dysfunction and so-called 'uraemia' are associated with the haemolytic crisis. The liver-copper concentration is usually higher than 1000 p.p.m. but an abnormally high liver value cannot be taken as proof of copper poisoning unless haemaglobinuria is also present associated with central necrosis of the liver lobules and with a premonitory rise in blood copper. Under field conditions the haemolytic crisis is commonly precipitated by a falling plane of nutrition, by fasting associated with movement or handling of stock, or by further assimilation of copper, when the liver-copper concentration is already high.

In Britain outbreaks of chronic copper poisoning in sheep associated with grazing in orchards where horticultural copper sprays were used have been reported by Fincham<sup>46</sup> and Ogilvie.<sup>47</sup> Weybridge records show that its occurrence under these conditions is much more frequent than published reports indicate, and confirm Boughton & Hardy's<sup>42</sup> observation that the fatal haemolytic crisis can occur some months after the sheep have been removed from the source of extraneous copper,<sup>48</sup> the precipitating factors being a falling plane of nutrition or other stresses. Clegg<sup>49</sup> reported chronic copper poisoning in sheep associated with the feeding of a copperized mineral supplement to grazing rams given in addition 2 to 3 lb. of a concentrate mixture per head per day. Pearson<sup>50</sup> described an outbreak caused by the feeding of a concentrate mixture containing approximately 0.07% of copper sulphate. Another unreported though confirmed outbreak in a pedigree flock investigated by Peggie<sup>51</sup> was associated with the feeding of a concentrate ration and a commercial copperized mineral mixture.

These reports fall under the first set of conditions described by Bull<sup>44</sup> and because of the widespread use of mineral mixtures in this country it is important to focus attention on them. A case which falls under the second set of conditions, i.e., chronic copper poisoning associated with a 'normal' copper concentration in the food, occurred in a group of eight stalled sheep at Weybridge fed for about 3 years on hay and a concentrate mixture of crushed oats, bran and linseed cake. This ration contained approximately 12 p.p.m. of copper, 1.1 p.p.m. of molybdenum and 0.7% of inorganic sulphate. The sheep were accidentally subjected to some



sudden and unaccustomed exercise, within 24 hours of which there were two clinical cases of typical chronic copper poisoning with liver copper values of 1000–3000 p.p.m. On slaughter, two other animals showed liver-copper concentrations of about 1000 p.p.m.

In another group of 6 sheep maintained on a similar diet for 2½ years, liver-copper values of 1000–2000 p.p.m. were found but these sheep were not subjected to any nutritional or physical stress before slaughter and no copper poisoning symptoms occurred. In both these cases the molybdenum content of the diet was within the 'normal' range and much higher than that of the pastures on which chronic poisoning occurs in Australia.<sup>44</sup> The inorganic sulphate content was also high and according to Dick's findings<sup>32</sup> should have been ample to allow the molybdenum to exert its full limiting effect on liver copper storage.

Bovines have been reported to be more resistant than sheep to repeated doses of copper sulphate. Cunningham<sup>52</sup> observed no ill effects from feeding two growing calves, seven months old at the beginning of the experiment, and two adult cows, 0.8 to 5 g. of copper sulphate daily for nine months or longer. Kidder,<sup>53</sup> however, produced chronic copper poisoning and death in a steer after about four months on a daily drench of 5 g. of copper sulphate.

Recent field reports and chemical analyses of tissues at Weybridge indicate that copper poisoning in cattle, especially in young calves, occurs more frequently than has hitherto been suspected. Deaths due to acute poisoning in 1–2-year-old animals have occurred after intravenous injection of too large a dose of copper sulphate solution, with symptoms resembling those described by Cunningham<sup>52</sup> and with a similar absence of high liver-copper concentrations. He has pointed out that diagnosis of acute poisoning must be made on symptoms and circumstantial evidence and that estimation of liver copper is of no diagnostic help. In chronic cases the syndrome is similar to that observed in sheep and liver-copper values are sometimes a very valuable aid in diagnosis. This was so in the case of the death of a yearling animal after administration of copper sulphate drenches followed by the use of a commercial copperized mineral supplement for some months after a diagnosis of copper deficiency on clinical symptoms alone. The most serious recent losses, however, have occurred in young calves approximately 2–4 months of age which have been reared on commercial milk substitutes to which small amounts of copper sulphate had been added to bring the total copper content to 60–100 p.p.m. (Shand,<sup>48</sup> Venn *et al.*<sup>54</sup>). The molybdenum and sulphate contents of these calf foods were about 0.8 p.p.m. and 0.5% respectively; these values fall within the range found for most feeding stuffs in this country and the sulphate figure is appreciably higher than Dick's<sup>32</sup> 'low normal'.

In the bovine as in man and the rat, rabbit, guinea pig and dog, maximum liver-copper concentrations occur in the new-born animal and decline during the suckling period. Cunningham<sup>7</sup> has shown that a physiological fall in liver-copper concentration occurs in cattle from birth to yearling stage followed by a subsequent rise in adult livers. The sheep is a notable exception and in this species the concentration tends to rise continuously after birth.

It is of interest that relatively small additions of copper to the food of the young calf over the suckling period can result in such a rapid and dangerous increase in liver copper.

In this paper, aspects of copper poisoning and of copper, molybdenum and sulphate contents of feeding stuffs have been discussed at some length as well as the occurrence and clinical syndromes of copper deficiency. This is because it is wished to emphasize the complexity of copper metabolism in cattle and sheep and to point out some of the complicated factors which must be taken into account when trying to make a diagnosis of, or recommend prophylactic treatment for, copper deficiency. Under field conditions the disorder is not confined to individual animals in a herd or flock and in our view it is not possible to arrive at an accurate diagnosis unless a representative number of samples are taken from the group of animals in question. Nor is it possible to assess the probable copper status of animals from chemical examination of samples of feeding stuffs or soils in this country. No surveys have been carried out here relating soil type to mineral deficiencies as have been reported in New Zealand (Cunningham<sup>35</sup> and Taylor, Cunningham & Davies<sup>55</sup>). Some of the complexities of the problem can be realized from the fact that severe copper deficiency occurs in cattle on one of the Ministry's experimental farms where pasture copper values range from 15 to 50 p.p.m.—a range similar to that on which copper poisoning occurs in sheep in Australia.<sup>44</sup> And 'swayback' has occurred frequently on a farm with pasture containing copper, molybdenum and inorganic sulphate values in the

vicinity of 12 p.p.m., 1 p.p.m. and 0.7% respectively while chronic copper poisoning developed in stalled sheep on a diet supplying similar concentrations of these constituents.

Because of these complications, it is suggested that copperized mineral supplements and 'shot-gun' trace-element mixtures should be used only in cases where they are known to be required. It has previously been thought that the amounts of copper generally used in copperized mineral supplements were insufficient to produce harmful effects, particularly in cattle because of the considerable margin of safety which was thought to exist between prophylactic or therapeutic levels and those likely to cause toxic effects. Present reports indicate that much more work is necessary before the copper requirements of animals under various conditions are known. It would seem that there is a strong case for revision of the present haphazard methods of mineral supplementation of rations now used so widely. Dick's recent work<sup>29-33</sup> showing the importance of the inter-relationships of copper, molybdenum and inorganic sulphate in feeding stuffs, points clearly to some of the complexities and should act as a deterrent to indiscriminate use of 'shot-gun' remedies.

## References

- <sup>1</sup> Neal, W. M., Becker, R. B., & Shealy, A. L., *Science*, 1931, **74**, 418
- <sup>2</sup> Sjollema, B., *Biochem. Z.*, 1933, **267**, 151
- <sup>3</sup> Bennetts, H. W., & Chapman, F. E., *Aust. vet. J.*, 1937, **13**, 138
- <sup>4</sup> Innes, J. R. M., & Shearer, G. D., *J. comp. Path.*, 1940, **53**, 1
- <sup>5</sup> Bennetts, H. W., & Hall, H. T. B., *Aust. vet. J.*, 1939, **15**, 152
- <sup>6</sup> Bennetts, H. W., *J. Dep. Agric. W. Aust.*, 1955, **4**, 42
- <sup>7</sup> Cunningham, I. J., *N.Z. J. Sci. Tech.*, 1946, Sect. A, **27**, 381
- <sup>8</sup> Cunningham, I. J., in 'Symposium on Copper Metabolism', eds. McElroy, W. D., & Glass, B., 1950, p. 246 (Baltimore: Johns Hopkins Press)
- <sup>9</sup> Allcroft, R., *Nature, Lond.*, 1946, **158**, 796
- <sup>10</sup> Allcroft, R., & Lewis, G., *Landbouwk. Tijdschr.*, 's Grav., 1956, **68**, 711
- <sup>11</sup> Wind, J., & Deijs, W. B., *Landbouwk. Tijdschr.*, 's Grav., 1952, **64**, 23
- <sup>12</sup> Neenan, M., Walsh, T., & O'Moore, L. B., *Proc. 7th Int. Grassland Congr., New Zealand*, 1956 (in press)
- <sup>13</sup> Ferguson, W. L., Lewis, A. H., & Watson, S. J., *J. agric. Sci.*, 1943, **33**, 44
- <sup>14</sup> Dick, A. T., & Bull, L. B., *Aust. vet. J.*, 1945, **21**, 70
- <sup>15</sup> Britton, J. W., & Goss, H., *J. Amer. vet. med. Ass.*, 1946, **108**, 176
- <sup>16</sup> Davis, G. K., in 'Symposium on Copper Metabolism', eds. McElroy, W. D., & Glass, B., 1950, p. 216 (Baltimore: Johns Hopkins Press)
- <sup>17</sup> Walsh, T., Neenan, M., & O'Moore, L. B., *Nature, Lond.*, 1952, **170**, 149
- <sup>18</sup> Cunningham, H. M., Brown, J. M., & Edie, A. E., *Canad. J. agric. Sci.*, 1953, **33**, 254
- <sup>19</sup> Russell, F. C., *Imperial Bureau of Animal Nutrition, Aberdeen*, 1944, Tech. Commun. No. 15
- <sup>20</sup> Green, H. H., *Brit. Commonw. Sci. Off. Conf., Spec. Conf. Agric. Aust.*, 1949 [1951] (London: H.M.S.O.)
- <sup>21</sup> Allcroft, R., *Vet. Rec.*, 1952, **64**, 17
- <sup>22</sup> Allcroft, R., Unpublished data
- <sup>23</sup> Allcroft, R., & Parker, W. H., *Brit. J. Nutr.*, 1949, **3**, 205
- <sup>24</sup> Jamieson, S., & Allcroft, R., *Brit. J. Nutr.*, 1950, **4**, 16
- <sup>25</sup> Marston, H. R., *Physiol. Rev.*, 1952, **32**, 66
- <sup>26</sup> Lee, H., *J. agric. Sci.*, 1956, **47**, 219
- <sup>27</sup> Stewart, J., Farmer, V. C., & Mitchell, R. L., *Nature, Lond.*, 1946, **157**, 442
- <sup>28</sup> Lewis, G., & Allcroft, R., *Proc. Nutr. Soc.*, 1953, **12**, (2), ix
- <sup>29</sup> Dick, A. T., *Aust. vet. J.*, 1953, **29**, 18
- <sup>30</sup> Dick, A. T., *Aust. vet. J.*, 1954, **30**, 197
- <sup>31</sup> Dick, A. T., *Aust. vet. J.*, 1954, **30**, 197
- <sup>32</sup> Dick, A. T., in 'Inorganic Nitrogen Metabolism', eds. McElroy, W. D., & Glass, B., 1956, p.445 (Baltimore: Johns Hopkins Press)
- <sup>33</sup> Dick, A. T., *Proc. 7th Int. Grassland Congr., New Zealand*, 1956 (in press)
- <sup>34</sup> Allcroft, R., & Lewis, G., *Proc. 7th Int. Grassland Congr., New Zealand*, 1956 (in press)
- <sup>35</sup> Cunningham, I. J., *N.Z. J. Agric.*, 1955, **90**, 196
- <sup>36</sup> Cunningham, I. J., in 'Advances in Veterinary Science', eds. Brandy, C. A., & Jungherr, E. J., 1955, Vol. II, p. 138 (New York: Academic Press)
- <sup>37</sup> Underwood, E. J., 'Trace Elements in Human and Animal Nutrition', 1956 (New York: Academic Press)
- <sup>38</sup> Dick, A. T., *Aust. J. agric. Res.*, 1954, **5**, 511
- <sup>39</sup> Mills, C. F., *Biochem. J.*, 1954, **57**, 603
- <sup>40</sup> Mills, C. F., *Brit. J. Nutr.*, 1955, **9**, 398
- <sup>41</sup> Mills, C. F., *Biochem. J.*, 1956, **63**, 190
- <sup>42</sup> Boughton, I. B., & Hardy, W. T., *Texas agric. Exp. Sta.*, 1934, Bull. No. 499
- <sup>43</sup> Albiston, H. E., Bull, L. B., Dick, A. T., & Keast, J. C., *Aust. vet. J.*, 1940, **16**, 233
- <sup>44</sup> Bull, L. B., *Brit. Commonw. Sci. Off. Conf., Spec. Conf. Agric. Aust.*, 1949 [1951] (London: H.M.S.O.)
- <sup>45</sup> Bull, L. B., Dick, A. T., Keast, J. C., & Edgar, G., *Aust. J. agric. Res.*, 1956, **7**, 281
- <sup>46</sup> Fincham, I. H., *Vet. Rec.*, 1945, **57**, 581
- <sup>47</sup> Ogilvie, D. D., *Vet. Rec.*, 1954, **66**, 279
- <sup>48</sup> Shand, A., Unpublished data
- <sup>49</sup> Clegg, F. C., *Vet. Rec.*, 1956, **68**, 332
- <sup>50</sup> Pearson, J. K. L., *Vet. Rec.*, 1956, **68**, 766
- <sup>51</sup> Pegg, W. W., Unpublished data
- <sup>52</sup> Cunningham, I. J., *N.Z. J. Sci. Tech.*, 1946, Sect. A, **27**, 372
- <sup>53</sup> Kidder, R. W., *J. Anim. Sci.*, 1949, **8**, 623
- <sup>54</sup> Venn, J. A. J., and others, Unpublished data
- <sup>55</sup> Taylor, N. H., Cunningham, I. J., & Davies, E. B., *Proc. 7th Int. Grassland Congr., New Zealand*, 1956 (in press)

**Discussion**

*Dr. F. E. Moon* (Fisons Ltd.): Can molybdenum produce an initial rise in blood copper due to the mobilization of liver reserves which is then followed by a decrease? The liver biopsy data did not show a drop in liver copper when molybdenum was administered, and this throws some doubt on the view that molybdenum mobilized the liver-copper reserve. Could the effect have been on the absorption of dietary copper?

*Dr. Allcroft*: There is a fall of about 30% in the liver-copper reserves of individual animals, but the reduction in the group average is small in relation to the scale of the graph. Scouring occurs, however, before the deficiency level is reached. There is usually an initial rise in blood copper when molybdenum is administered.

*Dr. E. J. Butler* (Moredun) commented briefly on the pathology of swayback. In Scotland the gross cavitation of the brain found by Innes in Derbyshire has been rarely seen. Frequently the demyelination is very slight, and the pathologists have great difficulty in observing it. In conjunction with Mr. Barlow of the Edinburgh and East of Scotland College of Agriculture, a study is being made of a central nervous disease of lambs, clinically resembling swayback, associated with a similar copper status, but histologically different from swayback. Demyelination is not a primary feature of this disease.

*Dr. Allcroft*: Cavitation was not common in Australia and New Zealand.

*Dr. Butler*: Motor degeneration may be a primary symptom. What are normal and low ranges for copper content of blood?

*Dr. Allcroft*: As the range of blood-copper values is very wide even in a flock of normal sheep with no history of swayback, a better measure of copper status is given by the liver-copper content where possible. Values below 20 p.p.m. on a dry matter basis can be regarded as low. In cattle, the blood-copper values of a representative number of animals gives a good indication of the copper status of the herd and values of 0.07–0.12 mg./100 ml. are taken as normal, values below 0.06 mg./100 ml. are low. Again, liver-copper content is a more reliable guide and values below 20 p.p.m. on the dry matter can be regarded as low.

*Dr. Robertson*: *Dr. Allcroft* has suggested that blood-copper levels in sheep of less than 0.06 mg./100 ml. would be suggestive of copper deficiency. In a study of a normal flock of 250 sheep of all age groups on a typical Scottish border pasture, a mean value of 0.036 mg./100 ml. was obtained, the range being 0.014–0.058. These values were obtained in April approximately 14 days before lambing, and any effect of low maternal copper on the foetus would have been apparent. All lambs were normal and there was no history of swayback on this pasture. With regard to scouring associated with teart, has molybdenum a physiological effect on the animal as a whole, is it associated with a change in the nitrogen metabolism in the rumen, or is it an effect on the xanthine oxidase activity of the intestinal mucosa?

*Dr. Allcroft*: We have not investigated the molybdenum effect in detail, but since injected molybdenum has produced scouring, it presumably had found its way into the intestine.

*Mr. G. Alderman* (N.A.A.S., Cardiff): In *Dr. Robertson's* paper on blood-copper levels in sheep, it is shown that the thyroid has a controlling effect on copper metabolism. Since the occurrence of swayback has been reported in ewes folded on kale, and as kale contains a goitrogenic factor, will *Dr. Allcroft* state whether a goitrogenic factor is the missing link in the copper metabolism story?

*Dr. Allcroft*: The evidence does not suggest that a goitrogen is the missing link, and although swayback does sometimes occur in lambs from ewes folded on kale, it is by no means an inevitable outcome of intensive kale feeding. Our investigations have shown clearly that marked enlargement of the thyroid glands of both ewes and lambs could result from intensive kale feeding without the occurrence of swayback. Although kale appears to exert a limiting effect on foetal liver-copper storage, our observations indicate that a low copper status alone does not necessarily result in swayback.

## THE INCIDENCE AND CONTROL OF COBALT DEFICIENCY UNDER VARYING SOIL AND PASTURE CONDITIONS IN CONNEMARA, CO. GALWAY\*

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Two separate incidences of cobalt deficiency in grazing stock are described, *viz.*, 'galar truagha' in cattle in the coastal area on a soil of calcareous sand, and 'summer pine' in sheep, especially recently weaned lambs, pastured on specific tracts of acid peat in the inland hill area. In addition, cattle on the 'summer pine' peat land are often affected by 'bris brón' shown to be aphosphorosis. The traditional method of avoiding these malnutritional syndromes is systematic transference of stock to different grazing areas. Controlled experiments on supplying cobalt to stock showed that drenching the animals with aqueous cobalt sulphate solution fortnightly is preferable on calcareous sandblown pastures, and top-dressing the pastures with cobalt sulphate is advocated for acid peatland.

### Introduction

For the cure or prevention of specific malnutritional syndromes in grazing stock, it has been a long-established practice in different parts of the world to transfer animals at intervals from one grazing area to another. For example, Hutcheon<sup>1</sup> in Cape Colony referred to the occurrence of a rheumatoid condition in cattle termed 'stywesiekte'—later shown to be aphosphorosis<sup>2</sup>—and to the beneficial effects noticed by the early Dutch settlers which resulted from a change of affected stock to known areas of recovery. Fraser<sup>3</sup> in Devonshire and Hogg<sup>4</sup> in the Scottish Borders recorded that a pining condition in sheep—since shown to be due to cobalt deficiency by Patterson<sup>5</sup> in Devonshire and by Corner & Smith<sup>6</sup> in the Scottish Borders—might be prevented or cured by moving the flocks to another part of the hillside or down to lowland grazings. In more recent years Boddie<sup>7</sup> has described the practice in islands of the Outer Hebrides of preventing the occurrence of pining in cattle by changing the animals at intervals from the sandy pastures near the sea to the peat pastures of the uplands. He recorded that the appearance of clinical pining on one of the smaller islands coincided with the division of the commonland into crofts, thereby limiting the area of grazing of an animal to that of its own holding and, as was found later, the pastures of several of these newly created holdings were cobalt deficient.

In 1950 a field investigation was commenced of the incidence of three different malnutritional syndromes which have been indigenous to the Connemara region of Co. Galway for some generations and which for many years past have been dealt with in the traditional manner by the stock owners, *i.e.*, by moving the animals at intervals from one grazing area to another. These syndromes are (i) marasmus in cattle grazing on certain sandblown pastures near the sea-shores and known locally as 'galar truagha', (ii) marasmus in sheep grazing on specific areas of peatland commonage and known locally as 'summer pine' and (iii) a rheumatoid condition in cattle grazing over areas of commonage and known locally as 'bris brón'.

The findings, obtained from therapeutic response by affected animals, from controlled feeding experiments carried out on a number of farms and grazing areas and supported by the analytical data presented in Table I, show that 'galar truagha' in cattle and 'summer pine' in sheep are in each case due primarily to cobalt deficiency, while 'bris brón', which will be referred to in this paper briefly in so far as it relates to an interchange of stock from one grazing area to another, is aphosphorosis in its clinical form.

### Description of the area

In Connemara, the mountainous coastal area forming the western part of County Galway, the farms are, with few exceptions, small, and apart from an acre or so of tillage are entirely in grass. The grazings of the area are of two contrasting types, (i) calcareous sandy pastures

\* Read at Symposium on Trace Elements in Soils, Plants and Animals, organized by the Agriculture Group and Bristol Section, 10-12 April, 1957

**Table I**  
*The incidence of cobalt deficiency in Connemara, Co. Galway*  
 Ranges of levels of calcium, phosphorus and cobalt in oven-dried samples of mixed herbage

Category of pasture	Area	Soil type and parent rock	Type of grazing	Type of sward	Ranges of levels of calcium, phosphorus and cobalt in oven-dried samples of mixed herbage			Soil samples (Walsh <i>et al.</i> ) <sup>8</sup>			Occurrence of malnutritional syndromes
					Ca %	P %	Co p.p.m.	Ca p.p.m.	P p.p.m.	Co p.p.m. in conc. HCl extract	
A	Cobalt-deficient pastures in the coastal area	Blown calcareous sea sand on granite and felsite rock	Enclosed fields	Varying from first-class rye grass-clover swards to agrostis-clover type swards	0.28	to	<0.04	0.0	to	7.5	Cattle maintained over a period on category A pastures may become affected with cobalt pine ('galar truagh'). Traditionally it is known that sheep cannot be kept on these pastures without serious losses from pining.
					0.37	to	<0.04	1.5	to	8.0	
B	Cobalt-deficient pastures in the inland hill area	Varied—mainly acid micaschist gneiss and quartzite	Commonage range (Semi-grazing)	Varying from agrostis-fescue type swards to mountain type herbage (heather, mat grass, cotton grass, sedges, flying bent, etc.)	0.06	to	0.12	0.3	to	3.9	Sheep only are affected with cobalt pine ('summer pine') on category B pastures. Cattle do not develop pining symptoms on these pastures
					0.12	to	0.15	2.5	to	5.3	
C	Pastures in the inland hill area which are reputed to be curative for pining in cattle or sheep	Peat on micaschist gneiss and quartzite	Commonage range (Semi-grazing)	Varying from agrostis-fescue type swards to mountain type herbage (heather, mat grass, cotton grass, sedges, flying bent, etc.)	0.16	to	>0.15	5.0	to	3.9	Cattle grazing for upwards of two months on many of the inland grazings (category B or C) may show clinical symptoms of aphosphorosis ('bris brón')
					0.16	to	>0.15	27.0	to	5.3	

of the coastal strip and the small inshore islands (Table I, category A) and (ii) acid peat pastures of farms and commonage in the mountainous hinterland (Table I, categories B and C).

(i) *Calcareous sandy pastures*

The soil of the coastal strip and inshore island area is derived predominantly from blown calcareous sand which has been deposited by action of the prevailing westerly winds. A brief description of the pasture swards is given in Table I (category A). These swards vary in quality; they are often relatively good, particularly if the soil has been tilled previously and large amounts of organic matter have been applied in the form of seaweed, peat mould or dung. Cattle only are maintained on many of the sandblown grazings and individual animals may develop symptoms of marasmus ('galar truagha') unless changed periodically to specific inland pastures. In many instances sheep rearing has been abandoned on these farms as a result of heavy mortality from pining in the past.

(ii) *Inland peat pastures*

Much of the hill and valley area of the hinterland is covered with varying depths of peat derived largely as a result of a high rainfall and humidity. The grazings of the small low-ground farms are utilized for the milch cows and the young stock while store cattle and sheep are pastured on the commonage. Sheep are kept mainly on the hill slopes and more particularly on the lower slopes as the upper slopes often consist of bare rock only. Although considerable variation exists in soil type from leached acid brown earths to podsoles and blanket peats,<sup>8</sup> peat is the material especially associated with symptoms of pine in sheep on inland commonage. Cattle do not develop symptoms of marasmus on the inland grazings and in fact may thrive reasonably well on pastures on which lambs become 'piners'.

A brief description of the swards of the inland mountainous pastures is given in Table I (categories B and C). The classification of the pastures is based on observations of the sheep-owners, category B referring to tracts of commonage on which 'summer pine' occurs annually in greater or lesser degree, while category C refers to non-pining or to reputedly curative areas. Category B and category C pastures are, in a few instances, adjacent one to the other (e.g., on different slopes or at different levels of the same hill) and it is rarely possible to delineate pining and non-pining areas by difference in the floristic pattern of the swards.

The Scottish Blackface breed of sheep is reared on the hillsides while a small hardy breed of cattle (Galloway-Aberdeen Angus type) and Shorthorn crosses are pastured on the commonage.

### Description of the syndromes

(i) '*Galar truagha*' is peculiar to cattle which have been kept over a period on certain sandblown coastal or island pastures (category A), the incidence being highest in young cattle up to 18 months of age. Initially, '*galar truagha*' is marked by loss of appetite, retardation of growth and progressive debility; in time this leads to depression, lethargy and complete cessation of grazing. The coastal farmers have known for many years that affected cattle recover rapidly if changed for a fortnight or so from the sandy pastures of category A to specific inland peat pastures of category C, which are known to be curative. In fact the traditional method of preventing the occurrence of '*galar truagha*' in cattle is by transference of stock for periods annually from sandblown coastal pastures to reputedly prophylactic inland grazings. As this method of prevention is practised widely the incidence of '*galar truagha*' is not high.

The sandy coastal pastures, while associated with the occurrence of '*galar truagha*' in young cattle, are known to be curative for '*bris brón*' (aphosphorosis) which is of common occurrence in cattle which have been pastured on many of the inland grazings (categories B and C). It has been the traditional practice to move affected animals to the coastal pastures where recovery follows within a few weeks. In fact it is not unusual for coastal and inland stockowners to exchange cattle for periods annually and thus control '*galar truagha*' in the animals belonging to the former and '*bris brón*' in those belonging to the latter.

(ii) '*Summer pine*' may occur in sheep grazing on 'affected' tracts of hillside or lowground commonage (category B pastures). The condition appears from late August onwards, the recently weaned lambs being more susceptible than the ewes or the previous year's lambs.

The clinical syndrome of 'summer pine' includes dejection, progressive weakness, pallid mucous membranes, ocular discharge and sometimes a broken fleece. A 'pining' lamb practically stops grazing and as a result loses flesh rapidly, becoming so weak that it staggers while walking and ultimately falls down and is unable to rise. Cobalt solution given orally produces a spectacular resumption of appetite and rapid improvement in condition in those animals which have not become moribund. In actual practice the owners of sheep grazing on known 'pining' areas of hill land (category B pastures) watch for symptoms of pine in their flock and know when to remove the animals to grazings which are reputed to be curative (category C pasture). The movement to fresh pastures takes place from late August onwards.

In some years the death rate in lambs from pining has been high during autumn and the early winter months; in other years losses have been negligible.

#### Investigational procedure

In August, 1950, it was observed that 'galar truagha' in cattle and 'summer pine' in lambs could be cured by the administration of small amounts of cobalt.<sup>9</sup> Accordingly, it was decided to make more detailed observations on a number of farms and areas of commonage. Included were five inshore island farms on which 'galar truagha' was evident in young cattle (category A), four areas of commonage on which there was a high incidence of 'summer pine' in the recently weaned lambs (category B) and five areas of commonage which were reputed to be curative for pining (category C).

Determination of total cobalt was carried out on a single sample of mixed herbage (grass species only) from each farm or area of commonage. During 1951 a further 140 herbage samples were collected and analysed for the proximate principles and for the following mineral elements: calcium, phosphorus, copper and molybdenum.

Blood samples were drawn from cows, calves and sheep at pasture in the various areas. Included were samples from animals showing clinical symptoms of 'galar truagha', 'summer pine' or 'bris brón'. Calcium, magnesium, inorganic phosphorus, copper and haemoglobin were determined on these samples.

#### Results

##### *Pasture samples*

The analytical data for calcium, phosphorus and cobalt are given in Table I. Copper values ranged from 7.5 to 14.6 p.p.m., while those for molybdenum ranged from 0.5 to 3.5 p.p.m. for peatland pasture and from 2.0 to 10.0 p.p.m. for calcareous sandy pastures (only 4% of the results for the latter were > 4.5 p.p.m.).

The data for the proximate principles presented no features of relevance to this investigation and they are not reported here.

##### *Blood samples*

The values for the constituents determined lay within the accepted normal ranges with the following exceptions:

Haemoglobin—in advanced cases of 'galar truagha' or of 'summer pine' haemoglobin levels were often < 6.0 g.-%.

Inorganic phosphorus—of 68 cattle grazing over peat pastures (categories B and C), 30 were found to have blood inorganic-phosphorus levels of < 2.5 mg.-%; 10 of these showed clinical symptoms of 'bris brón'. Hypophosphataemia under such circumstances is indicative of latent or of overt aphosphorosis.

#### Discussion of results

##### *Calcareous sandy pastures (category A)*

As will be seen in Table I, the level of total cobalt in each of five pasture samples collected from sandblown grazings is extremely low, i.e., < 0.04 p.p.m. Workers in Scotland and in Australasia have established that 0.07 p.p.m. of cobalt in dry pasture is the lower critical level for sheep. It has been known for many years that sheep cannot be reared on many of the sandblown pastures without heavy losses from pining, while cattle, if they are to remain healthy, must be changed periodically to specific inland peat grazings (category C).

The levels of phosphorus in herbage samples from category A pastures range from 0.28 to 0.37% of P (Table I). Such pastures provide adequate phosphorus for grazing cattle and, as already stated, the grazings of the coastal strip and inshore islands are known to be curative for 'bris brón' (clinical aphosphorosis). The copper contents of samples from these pastures are not abnormal; those for molybdenum are slightly above the normal range of 1 to 3 p.p.m.

#### *Inland peat pastures (categories B and C)*

The level of total cobalt in the pasture samples from category B areas varied from 0.06 to 0.12 p.p.m. as compared with > 0.15 p.p.m. in those from category C areas. A much clearer indication of the cobalt status of areas of commonage has been given recently by Walsh *et al.*<sup>8</sup> who report cobalt levels of < 2.5 p.p.m. in soil samples from 'pining' areas as compared with > 5.0 p.p.m. in those from reputedly curative areas.

The levels of phosphorus in herbage samples from peatland grazings (categories B and C) ranged from 0.12 to 0.16% of P. This is within the range associated with the occurrence of clinical aphosphorosis in other parts of Ireland.<sup>9, 10</sup> These herbage samples gave normal copper and molybdenum values.

#### Methods of control

Experiments were conducted to determine the preferable method of supplying cobalt to stock grazing on (a) calcareous sandy pastures of the coastal strip and inshore islands, and (b) peatland commonage of the hinterland. The methods compared were (1) oral dosage with aqueous cobalt sulphate solution at fortnightly intervals and (2) top-dressing areas of pasture with cobalt sulphate.

#### 'Galar truagha' (Experiment 1)

An inshore island farm on calcareous sandy soil was selected on account of its high incidence of 'galar truagha' in young cattle. In May, 1953, 30 Scottish Blackface ewes with their lambs were brought to this farm from the Co. Galway mainland. These were divided into three groups—10 ewes with their lambs in each group. The lambs were ear-tagged.

Group 1 was confined to grazing from which samples of mixed herbage were found to contain < 0.04 p.p.m. of cobalt. Each lamb received 14 mg. of cobalt in aqueous solution fortnightly.

Group 2 was confined to a grazing of similar cobalt status but which had been top-dressed with cobalt sulphate at the rate of 2 lb./acre.

Group 3 served as a control group and grazed on pasture of similar cobalt status, the lambs receiving no cobalt supplementation.

The ewes in each group received fortnightly an aqueous drench which supplied 14 mg. of cobalt.

The experimental period extended from the end of May until the third week of November and during this period the lambs were weighed fortnightly. All ewes and lambs received anthelmintic treatment on four separate occasions. The lambs were weaned and the ewes removed on August 15th.

*Results.*—(a) 'Pining'. The lambs in groups 1 and 2 remained healthy. Severe 'pining' occurred in five lambs in group 3. These recovered on being given cobalt orally.

(b) *Liveweight increases.* These are shown in Fig. 1. The mean increases at the end of the experimental period were: group 1, 47 lb.; group 2, 39 lb.; group 3, 21 lb.

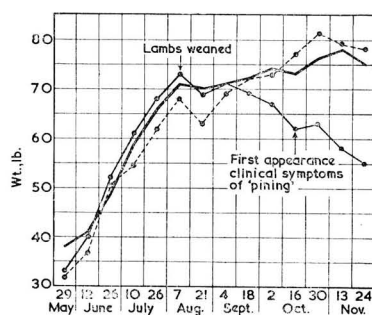


FIG. 1.—Mean liveweight gains (lb.) in groups of lambs grazing on cobalt-deficient calcareous sandy pastures

— Pasture dressed with 2 lb. of cobalt sulphate per acre  
 - - - - - Lambs drenched fortnightly with 14 mg. of cobalt (as Co) in aqueous solution  
 . . . . . Control group



## 'Summer pine' (category B pastures) (Experiment 2)

In May, 1953, a flock of 40 Scottish Blackface ewes with their lambs was divided into four groups—10 ewes with their lambs in each group. Each lamb was ear-tagged.

Group 1 was confined to a fenced-off tract of hillside, from which samples of mixed herbage were found to contain  $< 0.07$  p.p.m. of cobalt. The animals in this group received no cobalt supplementation.

Group 2 was confined to the same enclosure as group 1. These animals received 14 mg. of cobalt in aqueous solution at fortnightly intervals.

Group 3 was allowed to range over a category B pasture area on which 'summer pine' was of annual occurrence in the weaned lambs.

Group 4 was allowed to range over an area similar to that allotted to group 3. The animals had access, however, to half-acre plots which had been top-dressed with cobalt sulphate at the rate of 2 lb./acre using siliceous sand as a carrier.

The experimental period lasted from the end of May until the beginning of October and during this period the lambs in groups 1 and 2 were weighed fortnightly while those in groups 3 and 4 were weighed five times during the experimental period. All ewes and lambs received anthelmintic treatment on four separate occasions. The lambs were weaned and the ewes removed on August 15th.

*Results.*—(a) 'Pining'. The lambs in groups 2 and 4 remained healthy. By September 18th five lambs in group 1 were showing symptoms of 'pining'; these recovered following cobalt dosage. Two lambs in group 3 were found to be 'pining' when brought off the hillside on October 2nd. These also recovered following cobalt dosage.

(b) *Liveweight increases.* These are shown in Fig. 2. The mean increases at the end of the experimental period were: group 1, 7 lb.; group 2, 34 lb.; group 3, 13 lb.; group 4, 32 lb.

Essentially similar findings were obtained from replicates of this trial carried out at two other category B centres.

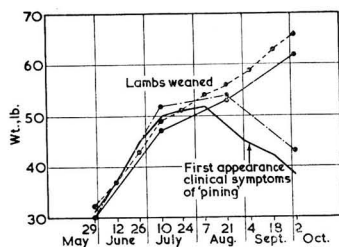


FIG. 2.—Mean liveweight gains (lb.) in groups of lambs grazing on cobalt-deficient hill grazings

Group 1. Confined to grazing containing 0.07 p.p.m. of Co  
 Group 2. Confined to similar grazing but receiving 14 mg. of cobalt fortnightly  
 Group 3. Allowed to range over hill grazings where 'summer pine' is of annual occurrence  
 Group 4. Allowed to range over a similar hill grazing but having access to plots dressed with cobalt sulphate

## General discussion

These investigations show that the pining conditions, 'galar truagha' in cattle and 'summer pine' in sheep, are due primarily to a deficiency of cobalt in the animal while 'bris brón' is clinical aphosphorosis. Accordingly it would seem that the transfer and interchange of stock which has long been practised in Connemara as a prophylactic measure, although empirical in nature, is fundamentally sound, being the result of intelligent observation by the rural community for many years past.

The fact that cattle may thrive on pastures on which sheep develop marasmus, and 'pine' only if confined to sandblown pastures on which, as has been known for many years, sheep cannot be reared without heavy mortality, confirms observations of other workers that the threshold level of cobalt in pasture is lower for cattle than for sheep.

With regard to methods of control, experiment 1 shows that it is possible to rear sheep on sandblown pastures by providing a source of cobalt. In this experiment it was found that satisfactory liveweight increases were obtained by supplying cobalt either by fortnightly drenching or by top-dressing the pasture. From the observations of other workers (e.g., Dr. A. B.

Stewart cited by Boddie?) it was thought that the gross excess of calcium carbonate in the soil might limit the availability of the cobalt applied to the pastures; however, although herbage cobalt analyses were not carried out, subsequent to top-dressing, the health and the liveweight increases in the lambs would suggest that this was not the case in this particular experiment. Nevertheless, until more is known of the uptake by different herbage species of cobalt applied to calcareous sandy soils, it has been recommended that the control of cobalt deficiency in stock in the coastal and island areas of Connemara should be by oral dosage. As the farms in question are small and the animals easily accessible, drenching once fortnightly should present no difficulty.

Experiment 2 and replicates carried out at two other centres show that cobalt supplementation either by drenching fortnightly or by top-dressing plots on the commonage prevents the occurrence of 'summer pine' and promotes liveweight increases in lambs grazing over tracts of land on which 'pining' is a problem annually. It was found that lambs having access to the cobalt-dressed plots made weight gains comparable with those receiving cobalt orally at fortnightly intervals. In Connemara the lambs, until they are sold, spend their entire lives on upland grazing, being gathered only for operations such as castration, dipping and anthelmintic treatment. Under such conditions of husbandry the drenching of all members of a scattered flock at fortnightly intervals would not be practicable. As has been found in areas of Great Britain, the top-dressing with a cobalt salt of a number of half-acre plots situated strategically is the preferable method of control of 'pine' under hill farming conditions.

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#### References

- <sup>1</sup> Hutcheon, D., *Agric. J. Cape of Good Hope*, 1894, **23**, 25
- <sup>2</sup> Theiler, A., Green, H. H., & DuToit, P. J., *J. Union S. Afr. Dept. Agric.*, 1924, **8**, 460
- <sup>3</sup> Fraser, R., 'General View of the County Devon', 1794 (London: Board of Agriculture)
- <sup>4</sup> Hogg, J., 'The Shepherd's Guide', 1807 (Edinburgh: Constable)
- <sup>5</sup> Patterson, J. B. E., *Nature, Lond.*, 1937, **140**, 363
- <sup>6</sup> Corner, H. H., & Smith, A. M., *Biochem. J.*, 1938, **32**, 1800
- <sup>7</sup> Boddie, G. F., *J. comp. Path.*, 1947, **47**, 52
- <sup>8</sup> Walsh, T., Fleming, G., Kavanagh, T. J., & Ryan, P., *J. Dept. Agric. Ireland*, 1956, **52**, in the press
- <sup>9</sup> O'Moore, L. B., *Vet. Rec.*, 1952, **61**, 475; *Proc. Symposium on Cobalt Deficiency in Ireland* organized by the Mond Nickel Co. Ltd., 1952
- <sup>10</sup> O'Moore, L. B., Thesis, Dublin Univ., 1950

#### Discussion

*Mr. Thomas*: Is there any correlation between pining with botanical factors? Have any of the pastures 20% or more of regularly burnt heather? In Northumberland, sheep are regularly walked from Scotland to graze on the heather.

*Dr. O'Moore*: In Connemara the occurrence of pining could not be correlated with the presence or absence of heather in a hill sward.

*Mr. Thomas*: In my experience I have never seen pining in the hill land in Northumberland when heather is present.

*Mr. J. B. E. Patterson* (N.A.A.S., Bristol): Contrary to the experience of Brynmor Thomas, the move is not to heather in Cornwall and Devon but to the red land. Fraser in 1794 reported that the same effect was obtained by drenching sheep with red loam. In the Bodmin Moor area the usual treatment was to top-dress one-quarter of the pastures every year with 2 lb. of cobalt salt per acre. In severe cases drenching was necessary.

*Dr. A. M. Smith*: In the past, red soil was often added to the sheep dip to show that sheep came from a red soil area.

*Mr. S. J. Cowlishaw* (Grassland Research Institute) : Did the worm drench contain copper and does heather grow on cobalt-deficient soil ?

*Dr. O'Moore* : The drench was phenothiazine and copper was not added. It is possible for heather to grow on cobalt-deficient peatland.

*Mr. A. D. Osborne* : Is there any correlation between the animals showing pinning and their weight at weaning ?

*Dr. O'Moore* : In the experiment carried out on calcareous sandy pasture the heavier lambs at weaning were those which developed symptoms of pinning.

#### General discussion

*Dr. E. J. Butler* : The point made by Allcroft & Lewis that a low copper status does not invariably lead to swayback should be emphasized. At the Moredun Institute we have found blood-copper levels with the swayback range in flocks with no history of the disease and when strict precautions have been taken to minimize any contamination. How does Dr. Allcroft define normal and low ranges of copper content of blood and liver of ruminants, and can she give any analytical data to amplify her statement that the concentration of copper in the liver of lambs rises continuously after birth ?

*Dr. Allcroft* : It is well known that in lambs there is a rise in level of copper concentration with age, whereas in cattle there is a physiological fall in the copper level up to yearling state followed by a rise in adult levels. In cattle and sheep there is a build-up of copper values on apparently normal rations, sometimes to toxic levels.

*Dr. Robertson* : Has Dr. Allcroft or any other member attempted to study the ratio of the different forms of copper in blood ? All the copper in blood is not present in the same form as there appears to be a fraction in serum capable of direct combination with diethyldithiocarbamate. A study of the ratio of this form to total plasma-copper might yield more valuable information than the figure for total blood-copper, particularly in the study of swayback.

*Dr. Butler* : At Moredun we are trying to evolve tests but have as yet little hope of success. *Dr. Allcroft* had no comment.

*Dr. J. W. S. Reith* (Macaulay Institute) : In N.E. Scotland we have found in the past 6-7 years a number of examples of copper deficiency in crops, and with copper addition we have obtained a crop response up to 100%. On these farms there is no evidence of any animal trouble associated with copper deficiency, although the pasture copper level is below 4 p.p.m. and generally between 2 and 3 p.p.m.

*Mr. W. H. Beaumont* : Can Dr. Allcroft give the relative incidence of diseases responding to copper administration and those due to chronic copper toxicity in ruminants. In the paper by Thompson on trace-element content of herbage, soil was rightly removed from samples before analysis. There is, however, a common farm condition called 'piglet anaemia', in which soil is deliberately given to the animal. In the light of work on the availability of iron from herbage, is any work being done on the availability of iron from the soil ?

*Dr. Allcroft* : Under field conditions there are very few instances of copper toxicity in this country, while there are many instances of copper deficiency.

**COBALT DEFICIENCY IN SHEEP IN HEREFORDSHIRE\***By **A. D. OSBORNE***University of Bristol, Veterinary Laboratory, Langford*

A pining condition of lambs associated with a deficiency of cobalt in the soil in an area in North Herefordshire is described. In this area, where stock normally thrive well, symptoms of failure to gain weight or actual loss of weight were noted in lambs between the ages of three and nine months but there was no indication that adult sheep or cattle were affected. The relationship between a variation noted in the severity of the condition in different years to the rainfall in the affected area during the period is discussed. Some evidence is presented that the symptoms are more marked when the temperature and rainfall combine to give a generous growth of pasture during the period of risk.

The relationship between cobalt deficiency and gastro-intestinal parasitism is considered and the suggestion made that deficient animals are more susceptible to the effects of parasites.

The greater effect of cobalt deficiency in the whole or neutered male as compared with the female is described and a comparison made with other observations on the effect of nutrition on the young growing male and female.

**Introduction**

This investigation into cobalt deficiency was made as a result of the observation on a single farm that a proportion of lambs, varying from year to year, failed to thrive during the summer and autumn.<sup>1</sup>

Chemical analyses of soils carried out on a large number of farms in Herefordshire and adjacent counties revealed that in a high proportion the cobalt content was equal to or lower than the critical level recorded by Patterson.<sup>2</sup>

The soils on all the farms sampled were relatively heavy clay from the Old Red Sandstone formation. The deficiency condition did not appear to be serious in the southern part of the county where the clay was lighter. Experiments were carried out to determine the response of lambs, in terms of weight gain, to the provision of cobalt sulphate given as a drench during the summer and autumn.

It was realized that, in dealing with the grazing animal, factors other than purely nutritive ones could be involved. In particular, the effect of parasitism and its relationship to pasture management were considered when pining was associated with diarrhoea. The importance of ensuring that experiments were not too much complicated by factors of this kind was realized when the parasitic ova content of the faeces of many lambs was checked and it was found that these gave evidence of significant worm infestation. The close interrelation of rainfall, growth of pasture, suitability for development of parasite larvae and possibility of variation in concentration of cobalt in the pasture was realized during the first three years of the experiments and some attempt was made to investigate this relationship during the next three years.

It was not until the third year of the experiments that the importance of sex was evident and measures adopted to investigate this feature more closely.

**Experimental**

In the main experiments two groups of lambs of approximately equal size and weight were used. To the members of one group a drench containing 14 mg. of Co as cobalt sulphate was administered once every fourteen days. The second group served as the control. As far as possible the two groups were mixed and run with the main flock in order to simulate normal conditions. In the earlier experiments no distinction was made between castrated males and females but later the groups were subdivided into equal numbers of males and females and chosen in such a way that the average weight of each half group was as near as possible the same. Where it was difficult to balance the weights of males and females in the same group every effort was made to balance the weights between groups. Strict randomization was not

\* Read at Symposium on Trace Elements in Soils, Plants and Animals, organized by the Agriculture Group and Bristol Section, 10-12 April, 1957

carried out since it had been noted that many of those lambs which were younger and smaller at the beginning of the experiment had suffered most from a deficiency of cobalt. Each sub-group therefore included some small lambs and some older, heavier animals.

In some experiments it proved possible to ensure a balance between the number of twin and single lambs in each group and thus obviate a factor of early nutrition which could have made a small difference in an area where the pining condition was not severe.

Each lamb was numbered for identification by branding with a special fleece-marking paint. The mark was found to last 2-4 months, was easily read at a distance and overcame the difficulty of earmarking black-eared sheep. With individually numbered lambs it was possible to note, and if necessary exclude from the experimental results, marked changes in weight resulting from causes other than cobalt deficiency, such as blowfly myiasis.

Faecal samples were taken from representative animals in each group and when parasite ova counts rose to levels suggestive of significant infestation appropriate anthelmintic treatment was given to the whole flock. Even when no marked evidence of parasitism was seen anthelmintics were administered at regular intervals of four to six weeks.

The experiments were begun not later than mid-July and finished in October or later.

**Results**

The results have been published in part elsewhere,<sup>1, 3</sup> The original data are presented in modified form with additional details in Table I.

**Table I**  
*Effect of cobalt on weight gain of lambs*

Farm and year	Sex	Group and no. in group	Mean of initial weights, lb.	Mean gain, lb.	Differences between means, lb.
P 1948	Mixed	Cobalt 20	69.8	21.7	14.90
		Control 18	66.0	6.8	
M 1950	Mixed	Cobalt 19	58.7	26.0	11.10
		Control 18	62.4	14.9	
N 1950	Mixed	Cobalt 20	72.15	17.3	6.30
		Control 18	76.5	11.0	
M 1951	Males	Cobalt 10	60.8	37.1	8.50
		Control 10	56.9	28.6	
	Females	Cobalt 10	55.7	31.5	2.60
		Control 10	59.0	28.9	
Total	Cobalt 20	58.25	34.3	5.55	
	Control 20	57.95	28.75		
N 1951	Mixed	Cobalt 15	63.3	38.7	- 1.5
		Control 15	66.1	40.2	
		Co/Cu** 14	68.8	44.0	
Tr 1951	Males	Cobalt 8	53.5	40.5	0.60
		Control 6	50.3	39.9	
		Co/Cu** 8	53.6	45.0	
	Females	Cobalt 12	50.5	36.75	- 0.20
		Control 12	51.3	36.95	
		Co/Cu** 12	57.75	37.75	
Total	Cobalt 20	51.7	38.2	0.2	
	Control 18	50.9	38.0		
	Co/Cu** 20	56.1	40.6		
V 1951	Males	Cobalt 8	61.6	39.1	6.85
		Control 8	61.0	32.25	
	Females	Cobalt 12	64.25	37.1	- 0.54
		Control 11	63.1	37.64	

Table I (continued)

Farm and year	Sex	Group and no. in group	Mean of initial weights, lb.	Mean gain, lb.	Differences between means, lb.
P 1951	Total	Cobalt 20	63.2	37.9	2.30
	„	Control 19	62.2	35.6	
	Males	Cobalt 10	67.0	22.6	- 0.1
	„	Control 10	66.3	22.7	
	Females	Cobalt 10	66.5	22.7	2.0
	„	Control 10	65.0	20.7	
Th 1952	Total	Cobalt 20	66.75	22.65	0.95
	„	Control 20	65.65	21.70	
	Males	Cobalt 10	67.4	41.0	8.80
	„	Control 11	70.9	32.2	
	Females	Cobalt 10	58.6	39.1	5.70
	„	Control 9	59.7	33.4	
L 1952	Total	Cobalt 20	63.0	40.05	7.30
	„	Control 20	68.85	32.75	
	Males	Cobalt 10	59.1	40.7	8.80
	„	Control 10	61.0	31.9	
	Females	Cobalt 10	54.5	31.7	0.70
	„	Control 10	57.0	31.0	
AN 1953	Total	Cobalt 20	56.8	36.2	4.75
	„	Control 20	59.0	31.45	
	Males (Rams)	Cobalt 10	68.9	25.3	9.50
	„	Control 9	69.1	15.8	
	Females	Cobalt 9	70.3	25.1	1.30
	„	Control 9	69.3	23.8	
L2 1953	Total	Cobalt 19	69.6	25.2	5.40
	„	Control 18	69.2	19.8	
	Males	Cobalt 10	55.5	32.9	16.00
	„	Control 10	56.4	16.9	
	Females	Cobalt 10	54.2	30.4	11.10
	„	Control 10	54.1	19.3	
N 1953	Total	Cobalt 20	54.85	31.65	13.55
	„	Control 20	55.25	18.1	
	Males	Cobalt 14	79.0	27.0	4.10
	„	Control 14	80.8	22.9	
	Females	Cobalt 16	69.6	23.8	0.70
	„	Control 15	70.8	23.1	
Total	Cobalt 30	74.0	25.4	2.40	
	Control 29	75.6	23.0		

Unless otherwise stated males = wether lambs

\*\* This group dosed at fortnightly intervals with proprietary pill stated to supply 32 mg. of cobalt, 63 mg. of copper, 200 mg. of iron and 32 mg. of manganese

The results showed a greater gain in weight of those lambs receiving cobalt than the controls and in no experiment was a loss of weight recorded for either group taking the period as a whole. Some lambs lost weight however and occasional deaths, almost always in the control groups, were recorded. Unless the carcass of such animals could be obtained for thorough post-mortem examination its details were excluded from the experiment in case the cause of death should not have been a direct result of cobalt deficiency.

When the results of the later experiments are considered the outstanding feature is the difference in the relative gains of males and females. This is emphasized when the results for experiments carried out on nine farms are grouped (Table II).

Table II

Sex	Cobalt		Control		Average difference in wt. gain, lb.
	Total wt. gain, lb.	Average, lb.	Total wt. gain, lb.	Average, lb.	
Males	3052 (90)	33.9	2310 (88)	26.2	7.7
Females	3047 (99)	30.8	2738 (97)	28.2	2.6

(Numbers in parentheses = number of animals involved)

### Discussion

Some consideration has already been given to most of the results described, particularly with regard to the statistical significance of the figures and the relationship of the results to cobalt levels of soil (Osborne *et al.*<sup>3</sup>).

Two main points, however, can well be considered in greater detail. In the first place the fact that the control groups gained in weight in every experiment must be emphasized if these results are to be compared with those described in other parts of the world. The maximum difference between groups at the end of four months of experiments was less than 20 lb. and in general less than 10 lb. where sexes are not considered separately. This is important from a practical point of view since many stock owners would not realize the significance of such a relative failure to gain weight when no experimental data were available. It is only when the deficiency produces a serious loss of weight or death of a number of animals that many stock owners become concerned. Nevertheless even a small difference of this magnitude can seriously affect the economy of a farm carrying a hundred or more lambs, for in addition to the increased value of the live animal there will also be a less obvious increase in carcass quality.

Further, in assisting the lambs to reach market weight in a shorter time the provision of cobalt can also increase the stock-carrying capacity of the land. On one of the farms in the experiments the flock has been increased from eight hundred ewes and lambs to over one thousand by the use of cobalt. Although some of this increase may be due to other causes, a significant proportion has certainly resulted from prevention of the specific deficiency.

The second main point which warrants further discussion is the effect of cobalt deficiency on the sexes. The fact that male lambs normally grow faster than females has been noted by many authors, particularly when considering the period taken to reach 60 lb. live weight (Underwood & Shier,<sup>4</sup> Thompson<sup>5</sup>). These authors noted also that in general single lambs grew more rapidly than twins and those heavier at birth took a shorter time to reach 60 lb. live weight. Thompson also made the observation that, when the diet was restricted, the difference between male and female lambs was much less pronounced. Palsson & Verges<sup>6</sup> in a more detailed experiment showed that although female lambs gained more than males in the first nine weeks, over a longer period the advantage was with the males provided the ration was on a high plane. When the ration consisted only of lucerne hay and straw without concentrates the female lambs gained almost as much as the males. When lambs on a low level of nutrition were changed to a high level at six weeks the males responded more than the females, and conversely.

It does not seem surprising therefore that the addition of cobalt to a diet partly deficient in this element should result in a greater response on the part of the males. Examination of graphs shows that the animals on a supplemented diet gained as would be expected at the age of three to seven months whereas those not receiving cobalt show no significant distinction between males and females. Stewart<sup>7</sup> reported failure to repeat these results in Scottish experiments and it would therefore be interesting to have results of similar experiments carried out in other countries.

Relatively few experiments could be carried out on the same farm in successive years, but in 1949 the results on two farms were so inconclusive that a repetition was arranged in

the two succeeding years. On these occasions significant results were obtained. When the rainfall figures, expressed as a percentage of normal for each month, are studied (Fig. 1) it will be seen that in 1949 the summer rainfall in the months June to September inclusive was well below average and in June, July and August below 50% of the average. In 1950 the latter period showed an excess of rain, and in 1951 it was excessive in August although deficient in June and July. The pasture was only stunted in 1949 and if the findings of McNaught & Paul<sup>8</sup> are substantiated rather than those of Maunsell<sup>9</sup> it would be simple to explain the poor results in this year. There does appear to be at least some comparison with the circumstances described by Lee<sup>10</sup> in certain parts of South Australia where symptoms are seen only in certain years and ewes are not affected.

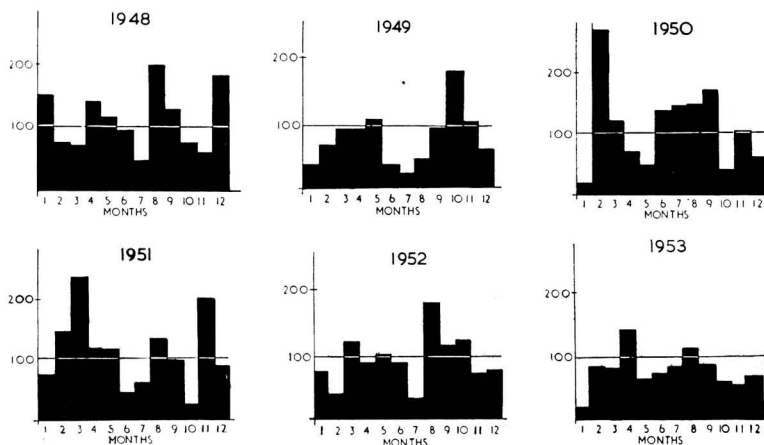


FIG. 1.—Monthly rainfall at Kimbolton 1948–53  
(expressed as a percentage of normal)

A recent report by Richard *et al.*<sup>11</sup> suggests that the addition of cobalt to a basal ration deficient in this and other elements results in a greater susceptibility of *Haemonchus contortus* infestation, but that when steamed bone flour was also added to the ration the resistance to the effects of worm infestation was maximal. During the experiments here described the results of worm egg counts and observations on the development of symptoms of parasitism in lambs showed that lambs on a cobalt-deficient diet were more likely to be affected by parasites. There was, however, no suggestion that the cobalt-supplemented animals were markedly resistant. In animals grazing intensively the best results were obtained when cobalt supplementation was combined with a regular and thorough programme of anthelmintic treatment.

### Conclusions

A moderate degree of cobalt deficiency exists in some parts of North Herefordshire and adjacent counties but it is not sufficient to cause extensive loss of condition in lambs. Male lambs appear to be more susceptible than female lambs to this degree of deficiency.

### Acknowledgments

This work was carried out with the full co-operation of Mr. J. Featherstone, Nutrition Chemist, West Midland Province, N.A.A.S., who was responsible for most of the soil sampling and analyses as well as helping with the animal experimentation. During the period much practical assistance was also given by Messrs. C. D. Rickaby and T. W. Griffiths. Mr. J. B. E. Patterson readily gave his advice in the planning of the experiments, none of which could have



been carried through completely without the co-operation of numerous veterinary surgeons and farmers in the area. The author is grateful for a small grant from the Ministry of Agriculture and Fisheries which was used in one experiment.

#### References

- <sup>1</sup> Osborne, A. D., Patterson, J. B. E., & Treanor, G. A., *Vet. Rec.*, 1950, **62**, 72
- <sup>2</sup> Patterson, J. B. E., *Nature, Lond.*, 1946, **157**, 555
- <sup>3</sup> Osborne, A. D., Featherstone, J., & Herdan, G., *Vet. Rec.*, 1954, **66**, 409
- <sup>4</sup> Underwood, E. J., & Shier, F. L., *J. Dep. Agric. W. Aust.*, 1942, **19**, 37
- <sup>5</sup> Thompson, D. S., *J. Dep. Agric. S. Aust.*, 1950, **53**, 352
- <sup>6</sup> Palsson, H., & Verges, J. B., *J. agric. Sci.*, 1952, **42**, 1
- <sup>7</sup> Stewart, J., Personal communication
- <sup>8</sup> McNaught, K. J., & Paul, G. W., *N.Z. J. Sci. Tech.*, 1939, (B) **21**, 25
- <sup>9</sup> Maunsell, P. W., *N.Z. J. Sci. Tech.*, 1945, (A) **27**, 40; *Nutr. Abstr. Rev.*, 1945/6, **15**, 432
- <sup>10</sup> Lee, M. J., Proc. of Specialist Conference in Relation to Soil and Climatic Factors, Australia, 1949, p. 262
- <sup>11</sup> Richard, R. M., Shumard, R. F., Pope, A. L., Phillips, P. H., Herrick, C. A., & Bohstedt, G., *J. Anim. Sci.*, 1954, **13**, 694

#### Discussion

*Mr. S. J. Cowlishaw* (Grassland Res. Inst.): Did the sheep have a heavy infestation and what were the egg counts in the faeces? What differences were there between dosed and undosed animals?

Mr. Osborne: We were unable to make as many observations as we wished, since the work was done at some distance from our headquarters. As a general rule however the worm egg counts in lambs not given cobalt were in the order of 1000 or more per g. whilst in the cobalt-drenched animals they remained at the 500 eggs per g. level or less. As the work was carried out on commercial farms it was not possible to carry out experiments in which a proportion of sheep were left without treatment for parasites.

*Mr. Patterson*: I saw the beginning of this work in 1948-49. Sub-clinical ill-thriving is of great financial importance to the farmers. In Herefordshire the reputation of the area for producing stock has been injured for this reason. One difficulty arose because the analytical data either for soil or for pasture did not give a very clear picture. Limits found acceptable elsewhere (Devon) could not be used with satisfaction in Herefordshire or Worcestershire.

## EFFECT OF ZINC, CALCIUM AND PHOSPHORUS ON THE SKIN AND GROWTH OF PIGS\*

By D. B. BELLIS and J. McL. PHILP

Unilever Ltd., Sharnbrook, Bedford

A dermatitis and growth syndrome occurring in 8–16-week-old pigs fed dry *ad lib.* on proprietary or home-mixed diets has been investigated and the clinical and histological observations described. Although mange mites were found in severely affected animals they were not responsible for the disease. The disease was aggravated by increased calcium and decreased by reduced calcium and increased phosphorus levels in the diet or by wet restricted feeding. Supplementation of the diet with 100 p.p.m. of zinc prevented or alleviated the condition with considerable increase in growth rate and efficiency. Zinc availability is apparently inversely related to that of calcium in the diet.

### Introduction

In 1953, Kernkamp & Ferrin<sup>1</sup> in the U.S.A. reported the incidence in pigs of a dermatitis termed parakeratosis which had been experienced for 11 years. They noted that the severity of the disease was increased by the addition of buttermilk to the diet. Thomas & Eden<sup>2</sup> also reported the occurrence of a skin disease of pigs in Britain in 1954 which they termed 'nutritional dermatitis', and noted that it occurred when pigs were fed dry *ad lib.* Unpublished reports (D. B. B.) indicated that the disease was widespread because this feeding method became popular during the post-war years, and in severe cases it resulted in considerable financial loss.

Tucker & Salmon<sup>3</sup> found that the dietary level of calcium and/or phosphorus affected the severity of the disease, but that it was cured or prevented by supplementation of the diet with 0.02% of zinc carbonate. Additional evidence that zinc has a beneficial effect on parakeratosis and that the incidence and severity of the disease is connected with the bone meal, calcium phosphate and limestone contents of the diet has recently been reported.<sup>4–6</sup>

The occurrence of dermatitis in pigs has seriously interfered with our feeding trials during the past few years and this paper presents the observations made when the disease was first encountered and during subsequent experimental work designed to investigate the effect on the disease of adding zinc and various levels of calcium and phosphorus to British-type pig diets.

### Effect of the dermatitis

In June 1953 it was noted that young intensively-housed pigs 8–16 weeks old receiving a commercial meal *ad lib.* developed a transient skin condition associated with poor growth. Litter mates on the same diet but limited to two wet feeds daily were not visibly affected.

The disease occurred with various home-mixed and commercially compounded diets and most cases were alleviated by changing the feeding of the animals on to a wet and restricted basis. The effect of the disease is illustrated in a trial which was carried out in August, 1953, using 80 eight-week-old weaning pigs, allocated into 5 blocks of 16. Within each block, 8 pigs were fed wet restricted, and 8 fed dry *ad lib.* The mean growths and skin conditions are compared in Table I.

Table I

Effects of method of feeding on incidence of dermatitis in pigs (40 animals)

Method of feeding	Average weight at 8 weeks, lb.	Average weight at 13 weeks, lb.	Rate of gain/day, lb.	Food conversion, 43–65 lb.*	Unaffected	No. of pigs Slightly affected	Severely affected
Wet restricted	43.2	77.4	0.98	2.91	36	4	—
Dry, <i>ad lib.</i>	43.3	66.7	0.67	4.22	12	18	10
Significant difference (P = 0.05)			0.19	0.62			

\* Mean amount of food (lb.) required for 1 lb. live-wt. gain over this growth stage

\* Read at Symposium on Trace Elements in Soils, Plants and Animals, organized by the Agriculture Group and Bristol Section, 10–12 April, 1957

Food conversion and daily growth were significantly poorer in the dry *ad lib.* fed group, which showed a marked incidence of the skin disorder. Lesions on the 'wet restricted' animals were faint and were seen only on close examination.

#### *Clinical observations*

Under ordinary farm conditions the disease would first be noticed as a skin condition, but when food intake and weight gains are recorded a gradual drop in appetite is revealed, followed a few days later by the first signs of skin affection—reddening of the skin of the belly and inner parts of the thighs, followed by the appearance of papular eruptions which develop a firmly adherent scab (see Fig. 1).

As the condition progresses the scab thickens, the lesions merge and in severe cases fissuring of the scabbed skin occurs. The hocks, knees, and hams are more commonly the sites of severe scabbing but around the ears and snout very painful though less scabbed lesions develop (Fig. 2).

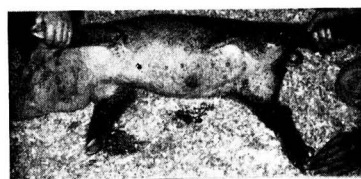
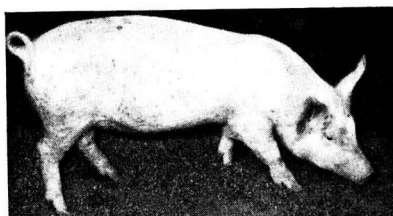


FIG. 1.—12-week-old large white pig showing early papular eruptions on hind legs, flank and ventral surface of the abdomen

FIG. 2.—12-week-old large white pig showing severe scabbing and fissuring of hocks, knees and hams

The hair becomes long and coarse and the pigs show discomfort by scratching and rubbing the affected areas. In affected groups there is considerable variation in the severity of the disease and its occurrence is not related to litter, sex or weight. Unless aggravated by diet or secondary infection the disease eventually disappears leaving rough, long coated pigs which may be 9–10 months old at bacon weight.

Clinically, the appearance of the skin suggests infestation by mange mites. This possibility was discarded after examination of early lesions by skin scrapings and serial sections from biopsy material had failed to demonstrate the presence of parasites, and also because precautionary treatment with acaricides failed to influence the course of the disease. Additions of vitamin concentrates and antibiotics to the diet, variations in the protein content and physical texture of the diet were tested but had no effect.

#### *Histological observations*

Attention was focused primarily on the early lesion to avoid confusing the histological picture with secondary bacterial, mycotic or parasitic invasion.

Histological examination of early lesions shows abnormal keratinization (parakeratosis) and acanthosis (Fig. 3).

The normal transition outwards from spinosum through granulosum cells to the clear anuclear corneum is replaced by an abrupt change from spinosum cells to a corneum largely composed of closely packed deeply staining nuclei. Some spinosum cells may show clear spaces in the cytoplasm and the nuclei appear compressed. Infiltrating leucocytes from the capillaries of the dermis are present and micro-abscesses filled with epithelial debris and leucocytes are formed below the abnormal corneum. Formation of further parakeratotic corneum beneath such abscesses gives an irregularly stratified appearance eventually lost in the dried mass of cell debris which forms the scab. The appearance of the dermo-epidermal junction is altered to a variable extent by irregular downward prolongations of the epidermis. Underlying the affected epidermis the dermis shows vascular dilatation and collagen bundles are separated by

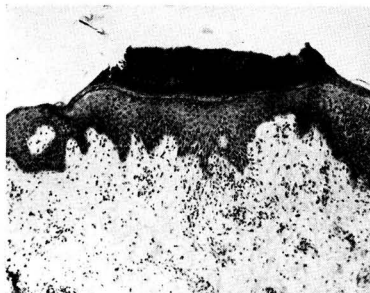


FIG. 3.—Section of skin showing early lesion. Parakeratosis and cellular debris forming a papular eruption can be seen. Acanthosis and irregularity of the dermo-epidermal junction are also present. Micro-abscess formation below the abnormal corneum can be seen towards the centre of the field

Magnification  $\times 65$

oedema. The upper third of the dermis is infiltrated with leucocytes; eosinophils are not common in early lesions. The epithelium of the perifollicular area often shows the most severe changes and the upper third of the hair follicles becomes filled with cellular debris but hair loss has not been noted.

Sections of skin from pigs with well-developed scabbing and fissuring may show occasional mange mites in the corneum or among the masses of cellular debris constituting the scab. Associated with their presence there is a great increase in cellular infiltration of the dermis. Many monocytes and eosinophils can be seen, eosinophils being particularly prominent in micro-abscesses adjacent to the parasite. The dermo-epidermal junction forms bizarre patterns within the upper third of the highly cellular oedematous dermis and intercellular oedema now becomes a feature of the epidermal change.

Consideration of the various aspects of the disease, particularly its apparent relation to dry feeding, suggested that it was nutritional in origin. Kernkamp & Ferrin<sup>1</sup> described a dermatitis in pigs under the title of parakeratosis and considered it might be due to a metabolic disturbance. Although only the major lesion of parakeratosis is mentioned by these authors the general description and course of the disease suggest that it is similar to the one under discussion.

In June, 1955, observations on a limited number of pigs on *ad lib.* feeding indicated that the disease was aggravated by the addition of bone meal to the diet. At the same time Tucker & Salmon<sup>3</sup> described trials indicating that bone meal influenced parakeratosis and that zinc had a beneficial effect.

### Effect of zinc supplementation of pig diets

#### *Experimental procedure*

In June, 1955, following reports of the beneficial effects of zinc on pig parakeratosis in the U.S.A., a trial was carried out to test the effect of adding 0.02% of zinc carbonate (100 p.p.m. of zinc) to the diet of young pigs, 8–16 weeks of age. Three treatments were tested as follows:

- A. Basal diet from 9 weeks (40 pigs)
- B. Zinc-supplemented basal diet from 9 weeks (40 pigs)
- C. Basal diet from 9–12 weeks, followed by zinc-supplemented diet (40 pigs)

The basal diet was typical of British compounded diets for pigs of 8–16 weeks of age (Table II).

The pigs were crossed Large White/Essex which had suckled their dams on pasture to 8 weeks of age. The age of the animals when the trial started varied from 8 to 9½ weeks, the older pigs being held on a commercial weaner meal until sufficient animals were available. They

Table II

Composition of basal diet (% by weight)			
Barley meal	45	Salt	$\frac{1}{4}$
Offals	12	*Trace minerals	$\frac{1}{2}$
Ground wheat	15	Limestone	2
Flaked maize	10	Bone meal	1
Fish meal	7	**Vitamin premix	$\frac{1}{4}$
Groundnut meal	7		

\* Premix containing 10% sodium ferrite, 2.5% copper sulphate, 2.5% manganese sulphate

\*\* Premix providing 9 million i.u. vitamin A, 1½ million i.u. vitamin D, and 1½ g. of riboflavin per ton of diet

Chemical analysis (% by weight)					
Oil	Protein	Fibre	Ca	P	Zn
2.3	17.2	4.3	1.32	0.70	22 p.p.m.

were allocated in pens of 8 to one of the treatments, with due regard to litter, sex and weight. Feeding was dry *ad lib.* through self-feeding hoppers and water was provided through automatic drinking bowls. The animals were weighed weekly and their food consumptions recorded.

Portions of skin for biopsy examination were taken from representative pigs when lesions were established and again after zinc supplementation. The skin sections were fixed in Bouin's fluid, followed by staining with haematoxylin and eosin; specific stains for bacteria and fungi were used where necessary.

### Results

At the beginning of the trial, close examination showed that a few of the pigs which had been held for 1½ weeks on unsupplemented diet showed faint traces of skin lesions but the skins and coats of the majority were in excellent condition. After 10 days on the trial, the appetite of all the pigs on the unsupplemented diets (A and C) dropped slightly and within another few days the dermatitis appeared with a marked decrease in appetite and rate of gain, and loss of condition. At 12 weeks, the severity of the disease varied considerably within a pen from only faintly visible skin lesions to extensive scabbing of the hind legs, groin, back and ears.

The effect of adding zinc to the diet of these pigs was dramatic. An improvement in appetite and condition was noted in 3–4 days. This was followed by a gradual loosening and shedding of the scabs and disappearance of the skin lesions. Recovery was complete within two weeks and there was a rapid improvement in weight gain.

Healing of the skin lesions was apparent histologically before full clearance would have been given on clinical examination. The most marked change was the reappearance of the granulosum layer and the formation of normal corneum free from nuclei (Fig. 4).

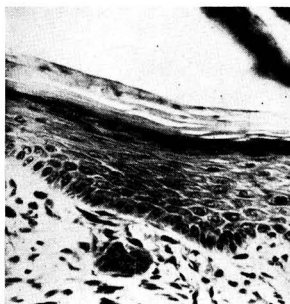


FIG. 4.—Section of skin from pig in Group C showing healing after zinc supplementation. A slightly thickened granulosum layer is present. Traces of abnormal corneum can still be seen and a portion of scab separated during sectioning is seen at the top right corner

Magnification  $\times 210$

The orderly arrangement of the epidermal strata was clearly demonstrable, infiltrating leucocytes were no longer present and the dermoepidermal junction had lost the irregular appearance previously seen in many cases.

It was to be expected that secondary bacterial, mycotic and parasitic infestation might occur as the nature of the lesions is such as to encourage their growth. Histological examination of one pig which appeared clinically to obtain no benefit from zinc supplementation showed widespread necrosis of the epidermis with secondary bacterial infection. In a section from a pig which responded to zinc and on which the lesions were healing a mange mite was seen but apart from the local reaction to its presence the healing did not appear to be affected.

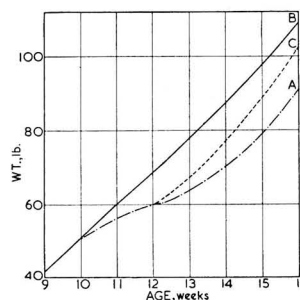
The health and condition of the animals on zinc-supplemented diet (B) from the start of the trial was excellent throughout: no loss of appetite was evident and growth rate was good. The skins of the pigs within this group which developed slight symptoms during the pre-trial period returned to normal within a few days. Careful examination of the animals in the group showed that no dermatitis developed during the experimental period, with the exception of one pig which showed very faint localized areas of scabbing; slight parakeratosis was confirmed by histological examination. The weight gains and food intake of this pig were in advance of those of its group and this finding may indicate a slightly higher requirement for zinc in this animal.

At 16 weeks, the majority of the animals on the unsupplemented diet (A) were slowly recovering from the disease. A few, however, were still very severely affected but they speedily recovered when zinc was added to the diet.

The relative growths of the pigs of the three groups during the experimental period is shown in Fig. 5 and a summary of food intakes and rates of gain in Table III.

FIG. 5.—Effect of zinc supplementation of pig diets

- A Unsupplemented diet from 9 weeks
- B Zinc-supplemented diet from 9 weeks
- C Zinc-supplemented diet from 12 weeks



When zinc was introduced into the diet at either 9 or 12 weeks of age it had a significantly beneficial effect on the rate of growth. These results are taken as evidence that the skin condition was related to sub-optimal levels of zinc in the diet and that adequate supplementation prevented or cured the disease. The view that the disease was nutritional rather than parasitic in origin was confirmed.

Table III

Effect of zinc on growth  
(Mean values/pig)

Treatment	9-12 weeks		12-16 weeks		9-16 weeks
	Rate of gain, lb./day	Food consumption, lb./day	Rate of gain, lb./day	Food consumption, lb./day	Rate of gain, lb./day
A. No zinc	0.81	2.80	1.14	3.67	1.00
B. Zinc at 9 weeks	1.23	3.50	1.49	4.50	1.38
C. Zinc at 12 weeks	0.81	2.80	1.54	4.60	1.23
Significant difference (P = 0.05)	—	—	—	—	0.18

**Effect of calcium and phosphorus levels in diets not supplemented by zinc***Experimental procedure*

This trial was carried out from January to June, 1956, with 262 8-week-old pigs and it consisted of seven diet treatments in two blocks. In the first block four levels of calcium were fed and in the second block three levels of phosphorus (Table IV). Diet No. 1 was identical with the basal diet (Table II) employed in the zinc trial, and all diets contained approximately 20 p.p.m. of naturally occurring zinc.

**Table IV**

<i>Composition of experimental diets (% by weight)</i>							
Diet :	1	2	3	4	5	6	7
Barley	45	45½	46¼	47	44½	43¾	43
Offals	12	12	12	12	12	12	12
Wheat	15	15	15	15	15	15	15
Flaked maize	10	10	10	10	10	10	10
Fish meal	7	7	7	7	7	7	7
Groundnut meal	7	7	7	7	7	7	7
Salt	¼	¼	¼	¼	¼	¼	¼
Trace minerals	½	½	½	½	½	½	½
Vitamin premix	¼	¼	¼	¼	¼	¼	¼
Limestone	2	1½	1¼	—	2½	1½	2½
Bone flour	1	1	1	1	1	2½	4½
Calcium (as Ca)	1·32	1·15	0·95	0·75	1·55	1·55	1·55
Phosphorus (as P)	0·70	0·70	0·70	0·70	0·70	1·00	1·30

Allocation of pigs to groups within a block, and their management, was identical with that in the zinc trial.

*Results*

The trial was completed when the pigs were 13 weeks of age in the 1st and 12 weeks in the 2nd block, and the results are summarized in Table V. The skin condition of the pigs at the end of this period is described as unaffected, slightly affected when a pink rash or early lesions were seen on the body, or severely affected when there was extensive scabbing.

**Table V***Effect of calcium and phosphorus levels on pig growth*  
(Mean values/pig)

Diet	% Ca	% P	No. of pigs	Wt. at 8 weeks, lb.	Rate of growth, lb./day	Food consumption, lb./day	Food conversion, lb.	Unaffected	No. of pigs Slightly affected	Severely affected
1	1·32	0·70	40	34·4	0·80	2·8	3·46	19	21	—
2	1·15	0·70	40	34·4	0·77	2·5	3·25	17	23	—
3	0·95	0·70	40	34·4	0·87	2·8	3·19	14	24	2
4	0·75	0·70	40	34·4	0·74	2·7	3·62	27	13	—
Significant difference (P = 0·05)					0·14					
5	1·55	0·70	34	38·0	0·77	2·8	3·61	8	14	12
6	1·55	1·00	34	38·0	0·63	2·4	3·78	8	20	6
7	1·55	1·30	34	38·0	0·63	2·5	3·87	15	14	5
Significant difference (P = 0·05)					0·10					

At 0·75% Ca (diet 4) there was a marked reduction in the number of affected pigs, but food consumption, food conversion and growth rate were not improved. All these figures were similar to those for calcium levels from ~1 to 1·3% (diets 1-3). There was a marked increase in the incidence of dermatitis with diet 5 (1·55% of Ca) but food consumption, food conversion and growth rate were not adversely affected over the experimental period. Increase in phosphorus from 0·7% to 1·3% (diets 5-7) reduced the incidence of dermatitis but food consumption, efficiency of food conversion and growth rate declined.

The zinc trial (see above) showed that the addition of zinc to a diet containing 1.3% of Ca and 0.7% of P not only prevented the onset of dermatitis but increased growth rate (Table III). The incidence of dermatitis in pigs fed diets 4 and 7 was much reduced but no comparable growth increase was obtained and it appears that the zinc content of these diets is not adequate.

Only a few calcium : phosphorus ratios have been investigated so far, and it is possible that further work may reveal a ratio that both prevents dermatitis and permits maximum growth.

### Discussion

It appears likely that the availability of zinc in a diet is inversely related to its calcium content. Such mineral inter-relationships have been frequently demonstrated and in many instances calcium appears in the dominant rôle. A similar relationship between calcium and phosphorus might explain the reduced incidence of dermatitis when the phosphorus level of the diet is increased ; in this case the phosphorus would be considered to have the effect of reducing the available calcium.

The changes noted in the skin resemble those described in studies of uncomplicated zinc deficiency in rats.<sup>7</sup> These authors also note extensive parakeratosis in the oesophagus of deficient rats and Follis<sup>8</sup> considers this to be unique among lesions caused by dietary deficiencies. Unfortunately it is not known whether this feature is present in the pig as no deaths occurred during the feeding trials and sacrificial post-mortem examinations were not carried out.

The amount of zinc required by the pig must be extremely low and critical if one assumes that interaction with calcium takes place at all levels of feeding. If this be so it might explain why a relatively slight increase in calcium in a diet containing naturally occurring zinc (20 p.p.m.) should bring about changes suggesting a zinc deficiency so rapidly. Pigs do not in our experience die from the disease but eventually recover after a period of poor growth. This again suggests a critical zinc requirement which alters as the demands for rapid growth are met. The reasons for the reduced incidence of the disease which occurs when wet feeding is practised are not clear, but are possibly related to the different food consumptions and hence growth potentials of pigs fed restricted and *ad lib.*, or to a different pH of the stomach under the two feeding methods.

The reaction of the skin, most notably parakeratosis, could be considered merely the non-specific reaction to stress ; there are indications however that stress inspired by various deficiencies produces different reactions. Parakeratosis is not associated with deficiency of vitamin A,<sup>9</sup> pyridoxine<sup>10</sup> or of essential fatty acids.<sup>9</sup> The reaction to external stress may well be more specific. Slight traces of parakeratosis may be seen in the skin of normal pigs and in such cases possibly represents the reaction to external stress similar to the hasty defence mechanism seen in man when the corneum is injured.<sup>11</sup> Personal observations (J. M. P.) in rats suggests that repeated external injury leads to exhaustion of the basal cells. However, in the sections of pig skin examined there was continual production of epidermal cells but the end-product, corneum, was abnormal. The view that keratinization is the result of a process of intrinsic differentiation<sup>12</sup> suggests a more direct relationship between zinc and this process of differentiation. Although no such relationship has been established, zinc is a co-factor for several enzymes and possibly continued work in this field may establish a closer link. Although the calcium content of the diet demonstrably affects the severity of the skin lesions, the mode of action is not necessarily the simple chemical one. The answer to this problem may well be found at the level of cell nutrition and growth in tissue culture. The relationship of such work to diet formulation is of fundamental importance. If this condition be truly a zinc deficiency the oft-repeated reference to 'there being no likelihood of such a deficiency occurring under practical feeding conditions' requires modification.

It is of some interest to note that since the work under discussion was carried out most commercial pig weaner rations have been adequately supplemented with zinc. What was once a disorder causing widespread concern where dry feeding systems were being used has now virtually disappeared. However, bearing in mind the constantly changing pattern of pig husbandry and feeding, it may be expected that similar critical relationships between constituents of the diet will become apparent, particularly while maximum food conversion and rapid attainment of carcass grade are accepted as criteria of progress.



### Conclusions

(1) There is little doubt that the disease here reported is similar to that observed in the U.S.A.<sup>1, 2</sup>

(2) The evidence indicates that the addition of 100 p.p.m. of zinc to the weaner meal employed (1.3% of Ca, 0.7% of P) will prevent or cure the dermatitis and give increased growth rate.

(3) Reduction of calcium or increase in phosphorus content of the diet will also reduce the incidence of the dermatitis, but growth rate and food conversion efficiency are not improved.

### Acknowledgments

The authors wish to record their thanks to the directors of Unilever Limited for permission to publish this paper, to Mr. G. C. Beveridge for the preparation and photography of histological material, and to Mr. J. A. Pratt and Mr. P. Holmes for the care of the experimental animals.

### References

- <sup>1</sup> Kernkamp, H. C. H., & Ferrin, E. F., *J. Amer. vet. med. Ass.*, 1953, **123**, 217
- <sup>2</sup> Thomas, G., & Eden, A., *Nature, Lond.*, 1954, **174**, 553
- <sup>3</sup> Tucker, H. F., & Salmon, W. D., *Proc. Soc. exp. Biol. Med.*, 1955, **88**, 613
- <sup>4</sup> Luecke, R. W., Hoefler, J. A., Brammell, W. S., & Thorp, F., *J. Anim. Sci.*, 1956, **15**, 347
- <sup>5</sup> Lewis, P. K., Hoekstra, W. G., Grummer, R. H., & Phillips, P. H., *J. Anim. Sci.*, 1956, **15**, 741
- <sup>6</sup> Stevenson, J. W., & Earle, I. P., *J. Anim. Sci.*, 1956, **15**, 1036
- <sup>7</sup> Follis, R. H., jun., Day, H. G., & McCollum, E. B., *J. Nutr.*, 1941, **22**, 223
- <sup>8</sup> Follis, R. H., jun., 'The Path of Nutritional Disease', 1948 (Oxford: Blackwell)
- <sup>9</sup> Ramalingaswami, V., & Sinclair, H. M., *Brit. J. Derm.*, 1953, **65**, 1
- <sup>10</sup> Sullivan, M., & Nicholls, J., *J. invest. Derm.*, 1949, **3**, 317
- <sup>11</sup> Pinkus, H., *J. invest. Derm.*, 1952, **19**, 431
- <sup>12</sup> Miszurski, B., *Arch. exp. Zellforsch.*, 1937, **20**, 122; cited by Rothman, S., 'Physiology and Biochemistry of the Skin', 1954 (Cambridge: University Press)

### Discussion

*Dr. D. P. Cuthbertson* (Rowett Institute): It would have been of interest to have been able to see a comparison between wet and dry diets of the same composition, where the total amounts of food fed were the same. Might the difference found be in part due to different rates of growth? In what main forms was the calcium fed: if it was as carbonate it might be of interest to recollect that Dr. Greig at the Rowett Institute has shown that excess of Ca (and Mg also) as carbonate, produced in rats a condition of iron deficiency possibly due to a neutralization of the gastric juice. Finally it is of interest to remember that zinc forms a constituent of most skin ointments and preparations used in human medicine.

*Mr. Bellis*: Wet feeding *ad lib.* was not practised because it is difficult to get the pig to consume all the food before it soured. We admit that there probably was a different growth potential. Calcium was supplied as calcium carbonate.

*Prof. H. C. Bendixson* (Veterinary School, Copenhagen): I note that the ration used was conspicuously rich in Ca and low in P, whereas in Denmark we feed a ration low in Ca but high in P. I have had some experience of what seemed to be a similar trouble, which occurred when a low-Ca ration was fortified by addition of calcium carbonate. The injury always occurred in the good, fast-growing pigs, and at 3-6 weeks after weaning. The onset of the malady was acute and the pigs looked as if they had measles. Thereafter a wet exudation appeared. If this was treated at once, cutting off calcium carbonate cured the trouble. My impression is that this was a complicated situation, involving nutritional balance.

*Mr. Philp*: I agree that this sounds like the same disease. Reducing the calcium content of the feed is a sound practice, as was indeed shown in our experiments, but in order to secure more rapid growth, the addition of zinc is necessary.

*Mr. Bellis*: The appearance of the disease depends on the age of the pig; generally at 11-12 weeks, independent of the age at weaning, whether 1 week or later.

*Dr. Mitchell* (Shinfield) : I have seen in the U.S.A. similar experiments in which all possible variations of wet and dry, restricted and unrestricted feeding were introduced. In agreement with what has been said today this work showed that injury was reduced only by the combined effect of wet feeding and restricted feeding.

*Dr. Owen* : With regard to the composition of the rations in Table II, does wet feeding inhibit development of parakeratosis because it enables phytase in the rations to hydrolyse the large amounts of phytic acid such as a seed-rich ration must contain? Phytic acid, if not removed in such a way, might precipitate zinc in unavailable form, and addition of calcium carbonate would only enhance this precipitation.

*Mr. Bellis* : In our experiments the food was wetted immediately before the pigs ate it and I doubt whether the phytase had more than a minute or two in which to act.

*Mr. Beaumont* : Has any work been carried out to demonstrate a growth-promoting effect of zinc when the diet is fed wet and restricted?

*Mr. Bellis* : This is being investigated now with a range of calcium-phosphorus ratios. No considerable effect has been found by adding zinc to the wet ration. In agreement, *Dr. Mitchell*, of Shinfield, remarked that this was the same as had been found in the U.S.A. experiments. That work was all done in wooden troughs, so that there was no risk of metallic contamination.

*Mr. G. Alderman* (N.A.A.S., Cardiff) : Is the addition of 2% limestone and 1% bone meal necessary to a ration containing 7% fish meal? Normally, fish meal as a pig ration constituent requires only 1% limestone and 1% salt addition. Has *Mr. Bellis* any information on the zinc content of pre-war pig meals?

*Mr. Bellis* : The ration was not one that I recommended. It was a commercial ration. In the third trial mentioned, the calcium content of the ration was considerably reduced, but nevertheless the parakeratosis effect was produced. The zinc content of pre-war and post-war meals were much the same, but the calcium in post-war diets was higher. Before the war, of course, there was no *ad lib.* feeding.

In another connexion we have tried massive doses of B vitamins, also vitamins A and D, but no curative effects have been achieved. In any case it is not to be expected that deficiency of B vitamin in pigs would produce this type of injury.

#### General discussion

*Dr. Owen* : Zinc deficiency might be expected to affect the rate of protein synthesis in view of the fact that zinc is an essential factor in carboxypeptidase.

*Mr. Beaumont* : It is a common practice, as a matter of convenience, to feed the same diet to the sow as to the weaned pigs. Is there any evidence to suggest that continuous administration of zinc in this way has any harmful effect on the sow?

*Mr. Bellis* : Little is known about this except that, in some experiments, massive doses of zinc given to sows have caused no injury. All the rations on sale within recent months have contained zinc, and there have been no reports of toxic effects.

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