Methylmalonic acid in the diagnosis of cobalt deficiency in barley-fed lambs

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Eight lambs were fed on a cobalt-deficient whole-barley diet supplemented with urea, vitamins and minerals. Four control lambs were fed on the same diet which had been further supplemented with Co. Plasma vitamin B_{12} levels in the Co-depleted group declined rapidly, falling below the normal range within 5 weeks. Differences between the live weights of the animals in the two groups approached statistical significance by week 14. However, methylmalonic acid (MMA) rose above normal levels in the Co-depleted group within 7 weeks. This suggested that an elevated plasma concentration of MMA is a comparatively early indicator of functional vitamin B_{12} deficiency. It is recommended that 10 μmol/l be the upper level of normality for plasma MMA concentration in barley-fed animals, in contrast with the level of 5 μmol/l for grass-fed animals. Changes in the plasma concentrations of MMA and ethylmalonic acid associated with feeding the barley-based diet *per se* did not significantly affect the validity of the gas–liquid chromatographic assay for MMA.

Cobalt deficiency: Methylmalonic acid: Vitamin B_{12}: Lamb

Vitamin B_{12} deficiency in ruminants occurs due to insufficient dietary intake of cobalt which is required by rumen bacteria for the synthesis of vitamin B_{12}. This vitamin, in the form of 5′-deoxyadenosylcobalamin, is a co-factor for the enzyme methylmalonyl-CoA mutase (*EC* 5.4.99.2) which converts L-methylmalonyl-CoA into succinyl-CoA. Vitamin B_{12} deficiency thus interferes with the production of succinate from propionate, the principal gluconeogenic precursor in sheep (Smith *et al.* 1967). This results in elevated tissue concentrations of methylmalonic acid (MMA) (Marston *et al.* 1961) which is a catabolic product formed by the hydrolysis of D-methylmalonyl-CoA, the immediate metabolic precursor of L-methylmalonyl-CoA (Kovachy *et al.* 1983).

Despite the widespread occurrence of Co–vitamin B_{12} deficiency in grazing sheep (Underwood, 1981), no consensus of opinion exists on the use of any single test for diagnosis of the disorder (Mills, 1981). A sensitive capillary gas–liquid chromatographic (GLC) assay for determination of plasma and urinary MMA was developed in this laboratory (McMurray *et al.* 1986). Using this assay it was shown that an elevated plasma MMA concentration (> 5 μmol/l) was a sensitive and early indicator of Co deficiency in grazing sheep (McMurray *et al.* 1985). That experiment demonstrated that as the animal's vitamin B_{12} stores became depleted, plasma MMA rose within about 8 weeks on Co-deficient pasture and continued to rise as the deficiency became more severe (McMurray *et al.* 1985; Rice *et al.* 1989). However, this was in sharp contrast to the work of Gawthorne (1968) who claimed that increased urinary MMA excretion occurred only in the later stages of deficiency.

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Table 1. *Components of complete diet (per kg)*

	Supplement
Whole barley	970 g
Urea	14 g
Vitamin A and D mix	10 mg (5000 and 1000 IU)
Vitamin E (50%)	40 mg (20 IU)
Calcium carbonate	15 g
Sodium sulphate (anhydrous)	400 mg
Zinc sulphate.$7H_2O$	150 mg
Manganous sulphate.$4H_2O$	50 mg
Potassium iodate (anhydrous)	1 mg
Sodium selenite (anhydrous)	175 μg
Cobalt acetate.$4H_2O$*	4·23 mg

Mineral supplements

* Added to control diet only.

As a consequence of the grazing trial we recognized the need for an experimental model system for examining the chronology of Co deficiency in greater detail. Such a model system could not make use of a grass diet since grass may have a variable Co content throughout the grazing season (Andrews, 1965). A complete barley-based diet (Table 1) similar to that described by Ørskov & Grubb (1977) was developed. The use of this diet permitted the re-examination of a report by Lough & Calder (1976) that increased urinary excretion of ethylmalonic acid (EMA) and MMA occurred in sheep fed on a barley-based diet. Although their assay was not sufficiently sensitive to measure EMA and MMA in plasma, their results imply that plasma MMA concentrations should also be elevated in barley-fed sheep. Confirmation of their observations by the demonstration of markedly increased concentrations of plasma EMA and MMA would preclude the use of EMA as an internal standard and the use of MMA in the diagnosis of Co deficiency in barley-fed sheep. Part of this work has been accepted for publication in a shortened form (O'Harte *et al.* 1989).

MATERIALS AND METHODS

Animals

Twelve Suffolk Cross lambs (ten females and two males) were weaned when 8 weeks old and adjusted to a concentrate diet by feeding sheep on pellets for 2 weeks before starting the experiment. The lambs were divided into two groups according to mean live weight values. One male lamb was placed in each group. The first group of eight animals was fed on a Co-deficient (4·2 μg/kg) ration and a parallel group of four controls was fed on the same ration with added Co (1000 μg/kg) for a total of 14 weeks.

Experimental diet

Both groups were fed *ad lib.* and had access to fresh water at all times. The components of the complete diet are shown in Table 1. All chemicals used were of GPR grade.

The mineral supplements (Table 1) sufficient for 40 kg diet were dissolved in distilled water (80 ml). This mineral solution was combined with 1120 ml urea (500 g/l) and sprayed onto the whole-grain barley (38·8 kg), while mixing. Vitamin E (1·6 g) and vitamins A and D (400 mg) were mixed thoroughly with calcium carbonate (600 g). This combination was then added slowly, with mixing, to the moist barley. Mixing was continued for 10 min after the final addition. The deficient diet was prepared in the same way except that cobalt

acetate was omitted from the mineral mix. The moisture content of the grain was increased by 3% after spraying. However, this did not have a detrimental effect on the subsequent storage of the complete diet.

Sample collection

Blood (30 ml) was collected from the jugular vein into lithium–heparin tubes at weekly intervals. Plasma, obtained by centrifugation at 2000 ***g*** for 20 min was stored at −20° until analysed.

MMA assay

Portions (250 μl) of plasma or MMA standards (100 μmol/l) were taken for determination of MMA as described earlier (McMurray *et al.* 1986). Calibration was carried out by comparison of peak areas of EMA and MMA in the standards and the final results expressed in μmol/l plasma. The limit of detection for plasma MMA was 0·2 μmol/l.

EMA assay

EMA levels were determined in 117 plasma samples collected from the present experiment. Group 1 represents the twelve pretreatment samples. The remaining samples were collected during the depletion experiment and were classified as Co-sufficient (group 2, *n* 55) or Co-deficient (group 3, *n* 50) on the basis of their plasma MMA concentrations (5 μmol/l was taken as the upper limit of normality for Co-sufficient animals). EMA was also determined in fifty-one randomly selected plasma samples from grass-fed lambs that had been received for diagnosis of Co deficiency from various parts of N. Ireland during the late summer of 1988. These were classified as Co-sufficient (group 4, *n* 32) or Co-deficient (group 5, *n* 19) using the same criteria as for groups 2 and 3.

Plasma EMA was measured using the existing capillary GLC MMA assay but without including EMA as internal standard. Standard solutions of EMA and MMA were prepared in the usual way, but sample EMA concentrations were calculated by comparison with the peak areas of EMA in the standards. The limit of detection for plasma EMA was 0·2 μmol/l.

Vitamin B_{12} assay

True plasma vitamin B_{12} analyses were performed using a commercially available radioassay kit (Becton Dickinson Immunodiagnostics, New York, USA).

Co assay

Determination of Co in feedstuffs was carried out on ashed samples using HGA atomic absorption spectrophotometry (W. J. Blanchflower and co-workers, unpublished results).

Statistical analyses

Analysis of the data relating to vitamin B_{12} and MMA concentrations was carried out using a one-tailed *t* test assuming that the variances were unequal. Analysis of the values relating to EMA concentration was carried out using a two-tailed *t* test assuming that variances were unequal. Live weight gains were analysed using a one-tailed *t* test assuming population variances to be equal.

RESULTS

Diet analysis

Table 2 shows the analysis of the complete diet into its major components and some minor minerals. The Co level was 4·9 μg/kg in the whole barley and 4·2 μg/kg in the Co-deficient diet.

Table 2. *Complete diet analysis (expressed as g/kg wet weight unless otherwise stated)*

Constituent	
Dry matter	847·5
Ash	34·5
Crude protein	150·1
Crude fibre	25·9
Oil	17·7
Gross energy	16·0 MJ
Sodium	0·4
Potassium	5·9
Magnesium	1·2
Calcium	5·2
Copper	5·9 mg
Selenium	0·08 mg

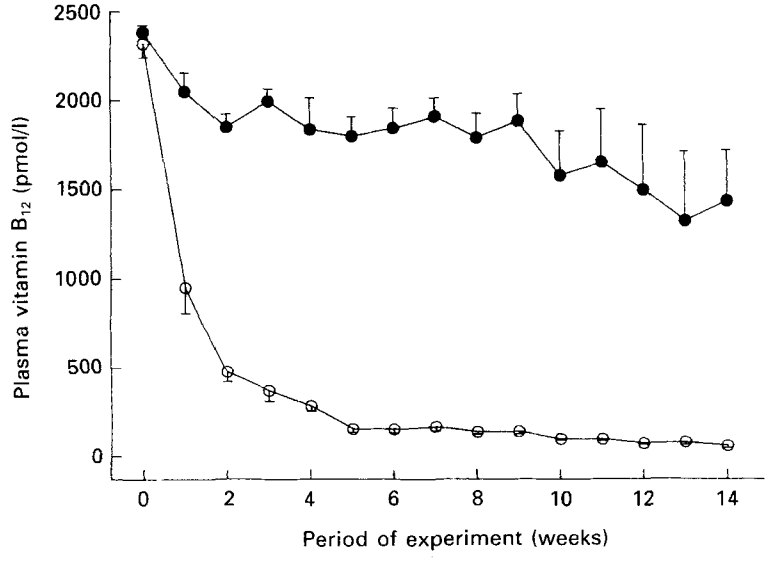

Fig. 1. Plasma concentrations of vitamin B_{12} in lambs fed on a cobalt-deficient (○, *n* 8) or Co-sufficient (●, *n* 4) whole-barley ration. Values are means with their standard errors represented by vertical bars. For details of dietary regimen, see p. 730 and Table 1.

Plasma vitamin B_{12}

Fig. 1 shows the plasma vitamin B_{12} results for both sets of lambs. Plasma vitamin B_{12} levels in lambs fed on the Co-deficient diet decreased sharply at first, falling below 220 pmol/l within 5 weeks. Lambs fed on the Co-sufficient diet maintained mean plasma vitamin B_{12} levels above 1300 pmol/l throughout the study. The differences between groups were statistically significant after 1 week ($P < 0{\cdot}01$).

Plasma MMA

Fig. 2 shows the plasma MMA levels in both sets of lambs. Mean plasma levels of MMA in lambs fed on the Co-deficient diet rose gradually, moving above 5 μmol/l within 6 weeks.

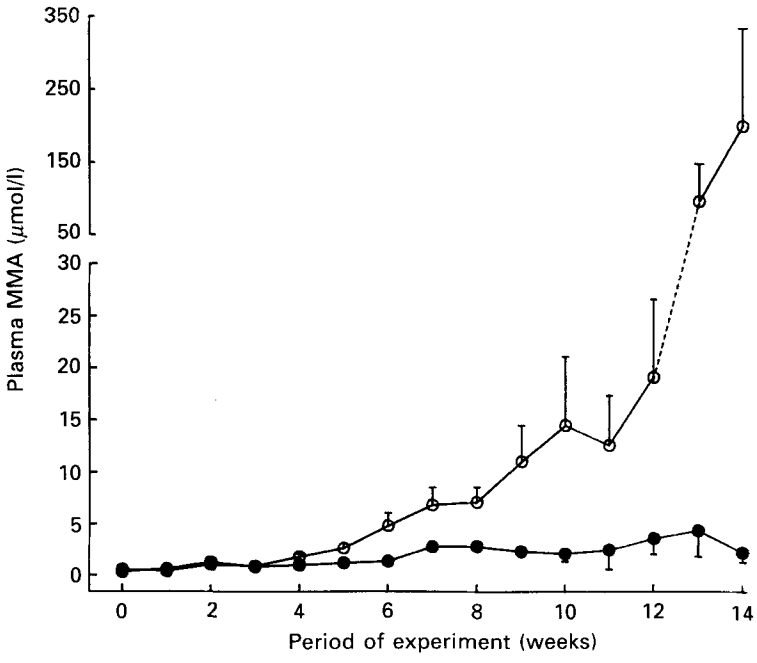

Fig. 2. Plasma concentrations of methylmalonic acid (MMA) in lambs fed on a cobalt-deficient (○, *n* 8) or Co-sufficient (●, *n* 4) whole-barley ration. Values are means with their standard errors represented by vertical bars. For details of dietary regimen, see p. 730 and Table 1.

There was a rapid rise between weeks 12 and 14. Intra-group variation was wide especially in the latter part of the present experiment. Mean plasma MMA concentrations remained below 5 μmol/l in the lambs fed on the Co-sufficient diet at all times; however, four individual results rose above this threshold between weeks 11 and 13.

Vitamin B_{12}–MMA inter-relationships

A scatterplot of all individual plasma MMA *v.* vitamin B_{12} results from the present experiment was constructed (Fig. 3). The plasma MMA levels ranged from 0·2 to 1120 μmol/l and vitamin B_{12} levels from 24 to 2550 pmol/l. Fig. 3 is dissected into quartiles using the limits of normality for both variables in grazing sheep (McMurray *et al.* 1985). The thresholds used are 220 pmol/l and 5 μmol/l for plasma vitamin B_{12} and MMA respectively. Results indicative of clinical normality are in the lower right-hand quartile. Results that probably indicate clinical and sub-clinical Co deficiency are in the upper left quartile.

Live weight

Fig. 4 shows the live weight changes in both sets of animals. The difference between the live weights of the control and deficient animals approached statistical significance by week 14 of the study ($P = 0{\cdot}057$). The live weight gains of Co-deficient and Co-sufficient lambs were 173 and 257 g/d respectively over the course of the experiment.

EMA

The concentration of EMA in the plasma of lambs at the start of the experiment was less than 0·2 μmol/l (Table 3). After the animals were fed on both experimental diets, plasma

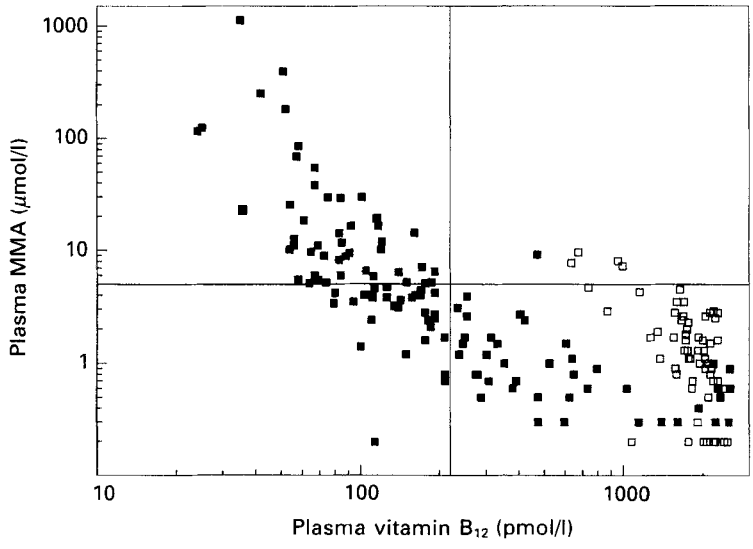

Fig. 3. Scattergraph of individual plasma methylmalonic acid (MMA) *v.* vitamin B_{12} values on logarithmic scales in lambs fed on a cobalt-deficient (■) or Co-sufficient (□) whole-barley ration. Thresholds of normality are set at 5 μmol/l and 220 pmol/l for plasma MMA and vitamin B_{12} respectively. For details of dietary regimen, see p. 730 and Table 1.

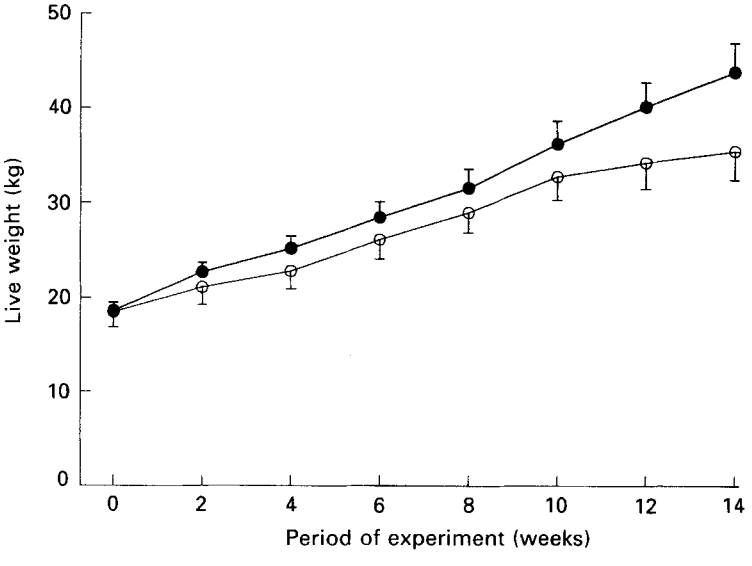

Fig. 4. Mean live-weight values for lambs fed on a cobalt-deficient (○, *n* 8) or Co-sufficient (●, *n* 4) whole-barley ration. Values are means with their standard errors represented by vertical bars. For details of dietary regimen, see p. 730 and Table 1.

Table 3. *Concentrations of methylmalonic and ethylmalonic acids in plasma of sheep fed on barley or grass diets with or without adequate cobalt**

Group no.	Dietary treatment	*n*	Plasma methylmalonic acid (μmol/l)	Plasma ethylmalonic acid (μmol/l)		Range (μmol/l)
				Mean	SD	
1	Pretreatment	12	< 5	< 0·2	—	—
	Barley diet:					
2	Co-sufficient	55	< 5	6·48	3·58	0·2–14·3
3	Co-deficient	50	> 5	6·02	3·43	0·2–14·7
	Grass diet:					
4	Co-sufficient	32	< 5	1·23	2·41	0·2–13·3
5	Co-deficient	19	> 5	0·89	0·73	0·2–2·3

Mean values were significantly different: group 1 *v.* groups 2+3 ($P < 0·001$), group 2 *v.* group 3 ($P > 0·1$), group 4 *v.* group 5 ($P > 0·1$), group 2 *v.* group 4 ($P < 0·001$), group 3 *v.* group 5 ($P < 0·001$).

* For details of dietary regimens, see p. 730 and Table 1.

EMA concentrations rose significantly (group 1 *v.* groups 2 and 3, $P < 0·001$). However, the differences in EMA concentration in plasma of Co-sufficient *v.* Co-deficient animals were not statistically significant (group 2 *v.* group 3, $P > 0·1$). Table 3 also shows the plasma concentrations of EMA in grazing sheep that were both Co-sufficient and Co-deficient. As with the barley-fed animals, there was no statistical difference between these groups (group 4 *v.* group 5, $P > 0·1$). However, animals fed on the barley-based diets did have higher plasma EMA concentrations than did the grazing animals ($P < 0·001$), irrespective of their Co status.

DISCUSSION

Diet analysis

Whole-grain diets with pelleted supplements containing proteins, minerals and vitamins are widely used for lambs. Urea, which is a suitable alternative to protein as a nitrogen source in ruminants (Helmer & Bartley, 1971), was added to the diet because of the difficulty of finding protein supplements with a low Co content. It is possible completely to adsorb urea into whole barley when it is added as a saturated solution without urea crystals re-forming on the outside (Ørskov *et al.* 1974). There are two main advantages of this approach. First the animals are unable to select particular constituents and second the absorbed urea gives slower release of ammonia in the rumen than does urea added as crystals.

Dietary analysis (Table 2) quantifies the major components of the diet. It is clear that only Co is deficient in this diet. The Co level in the whole barley (4·9 μg/kg) was reduced slightly following addition of the minerals and vitamin supplements required to produce the complete Co-deficient diet (4·2 μg/kg). This fall may be partly explained by the dilution effects of preparing the complete diet and by the addition of more moisture, but is likely to be within experimental error. The Co level in this diet is considerably lower than that reported for other Co-deficient diets (28–40 μg/kg) by Gawthorne (1970), Mann *et al.* (1983), Bremner *et al.* (1988) and Field *et al.* (1988).

Plasma vitamin B_{12}

Plasma vitamin B_{12} (Fig. 1) levels fell rapidly in lambs fed on the Co-deficient ration (Co 4·2 μg/kg). Plasma vitamin B_{12} levels fell below the threshold of impending Co deficiency for grazing sheep (220 pmol/l; McMurray *et al.* 1985) indicating that the lambs became deficient within 5 weeks.

Serum vitamin B_{12} concentrations on their own are not sufficiently specific to permit an accurate diagnosis of a functional deficiency of this vitamin (Suttle, 1986). Both the present study (Fig. 3) and that of Rice *et al.* (1987) show that animals may be vitamin B_{12} deficient yet have normal methylmalonyl-CoA mutase function, as judged by plasma concentration of MMA.

The mean plasma vitamin B_{12} levels in the lambs on the control diet (Co 1000 μg/kg) showed a gradual but distinct fall throughout the experiment. This is surprising considering the more than adequate dietary Co supply. It is known that efficiency of conversion of dietary Co to 'true' vitamin B_{12} is inversely related to Co intake (Gawthorne, 1970). Under present conditions of excess Co supply, it is possible that increased rumen production of cobalamin analogues occurs which may interfere with the absorption of 'true' vitamin B_{12}.

Plasma MMA

Plasma MMA in lambs fed on the Co-deficient diet showed an inverse relationship with plasma vitamin B_{12}. Plasma MMA concentrations in Co-deficient animals began to diverge from those in Co-sufficient animals after 4 weeks (Fig. 2). Previous studies with grazing sheep showed that plasma MMA levels in excess of 5 μmol/l were indicative of impending Co deficiency (McMurray *et al.* 1985; Rice *et al.* 1989). Mean plasma MMA concentrations in the Co-depleted lambs rose slowly during the initial stages of the experiment, moving above 5 μmol/l after week 6 and increased most rapidly from week 12 to week 14. There was a 1 week interval between the occurrence of low plasma vitamin B_{12} (< 220 pmol/l) and elevated plasma MMA (> 5 μmol/l) which may be due to the slower depletion of liver vitamin B_{12} stores (Sutherland, 1980). That plasma MMA levels rose above the upper limit of normality within 7 weeks in the present study is in marked contrast with the results reported by Gawthorne (1968). He suggested that the increase in urinary excretion of MMA was a very late manifestation of cobalt–vitamin B_{12} deficiency in sheep. In that study animals were fed on a Co-deficient diet for approximately 25 weeks before a significantly increased urinary excretion of MMA was observed. Thus, the present experiment together with the earlier reports from this group (Rice *et al.* 1987, 1989) suggest that changes in plasma MMA provide an early indication of the development of cobalt–vitamin B_{12} deficiency. Furthermore plasma MMA, unlike plasma vitamin B_{12}, is an active marker of Co deficiency because it directly reflects the functional vitamin B_{12} status of tissue.

The plasma concentration of MMA in lambs rose significantly (from 0·33 to 1·30 μmol/l, $P < 0{\cdot}05$) within 2 weeks of being fed on the Co-sufficient barley-based diet. This is in agreement with findings of Lough & Calder (1976) who showed that urinary concentrations of MMA increased when lambs were fed on a barley-based diet. However, the present study showed that the mean plasma MMA concentration did not continue to rise significantly when lambs were fed on the diet for longer periods. Despite this, four individual results (Fig. 3) exceeded 5 μmol/l (the upper limit of normality in grazing sheep), but none exceeded 10 μmol/l.

Vitamin B_{12}–MMA inter-relationships

Fig. 3 indicates the relationship between individual vitamin B_{12} concentrations and their corresponding MMA concentrations. The relationship is broadly similar to that observed

by Rice *et al.* (1987) who carried out a similar study using sheep grazing a Co-deficient pasture. In the present study, all the samples derived from the control animals had a normal plasma vitamin B_{12} concentration. The plasma samples located in the lower left quartile (17% of total) showed low plasma concentrations of vitamin B_{12}. However, the presence of a functional deficiency of vitamin B_{12} was not indicated, since their corresponding MMA concentrations were normal. Diagnosis of a functional deficiency of vitamin B_{12} on the basis of measurement of the vitamin B_{12} concentrations would have been incorrect. The presence of several samples in the upper right quartile, indicating an apparent functional deficiency of vitamin B_{12} in the presence of ample quantities of the vitamin, argues that the upper level of normality of MMA in barley-fed animals should be increased to 10 μmol/l. This contrasts with the level of 5 μmol/l suggested by McMurray *et al.* (1985) for grass-fed animals. Raising the upper threshold as suggested would eliminate the possibility of a false positive diagnosis of Co deficiency based on plasma MMA results alone. Samples located in the upper left quartile show signs of functional vitamin B_{12} deficiency, that is, a low plasma vitamin B_{12} concentration and an elevated plasma MMA concentration.

Live weight

The live weight gain achieved using the Co-sufficient diet in the present study (257 g/d) compares favourably with the value of 253 g/d reported by Ørskov & Grubb (1977). The mean weight of animals being fed on the Co-deficient diet was not significantly different from the controls throughout the study. However, the differences approached statistical significance by week 14 ($P = 0{\cdot}057$, Fig. 4). Nonetheless, their live weight gain was only 173 g/d. Since the Co concentration is the only difference between the two diets, it may be concluded that the reduced live weight gain observed here was due to a lack of dietary Co, and that changes in live weight are a late response to Co deficiency.

EMA

EMA was used as an internal standard in the plasma MMA assay (McMurray *et al.* 1986) because it has chemical characteristics similar to MMA, thus enabling the more accurate determination of MMA. However, the validity of this assumption depends on there being little or no endogenous EMA present in plasma samples. Lough & Calder (1976) claimed that elevated urinary excretion of EMA occurred in barley-fed sheep, thus implying that plasma EMA levels were also elevated. Pretreatment plasma EMA concentrations ($< 0{\cdot}2$ μmol/l) increased significantly to about 6 μmol/l when the animals were fed on the whole-barley diets ($P < 0{\cdot}001$, Table 3). However, the mean increases in plasma EMA were similar for both Co-depleted and control animals ($P > 0{\cdot}1$). The observation by Lough & Calder (1976) that feeding a barley-based diet resulted in elevated urinary excretion of EMA is supported by the present study. Significantly greater concentrations of EMA were detected in the plasma of barley-fed as opposed to grass-fed animals, irrespective of Co status ($P < 0{\cdot}001$). The mean plasma concentrations of EMA in barley-fed lambs, irrespective of Co status, were about 6 μmol/l (Table 3). However, since EMA is included as an internal standard in the MMA assay at a concentration of 200 μmol/l, the endogenous plasma EMA in barley-fed lambs will lead to an underestimation of plasma MMA levels by approximately 3%. This error is well within the experimental errors associated with this assay. Therefore, it may be concluded that the elevation in plasma EMA concentrations associated with feeding a barley diet does not preclude the use of EMA as an internal standard in the MMA assay.

In conclusion, previous work has shown that elevated plasma MMA taken in conjunction with decreased plasma vitamin B_{12} is a indicator of functional Co-deficiency in lambs fed on a grass-based diet. The present work has demonstrated that the same criteria can be used

for diagnosis of the disease in barley-fed animals. It is also suggested that the dietary regimen presented provides a useful model system for examining the chronology of Co deficiency under controlled conditions in sheep.

REFERENCES

Andrews, E. D. (1965). Observations on the thrift of young sheep on a marginally cobalt-deficient area. *New Zealand Journal of Agricultural Research* **8**, 788–817.

Bremner, I., Humphries, W. R., Morrice, P. C. & Carlyle, W. W. H. (1988). Control of selenium and cobalt deficiencies in lambs by supplementation with oral anthelmintics. *Veterinary Record* **123**, 217–218.

Field, A. C., Suttle, N. F., Brebner, J. & Gunn, G. W. (1988). An assessment of the efficacy and safety of selenium and cobalt included in an anthelmintic for sheep. *Veterinary Record* **123**, 97–100.

Gawthorne, J. M. (1968). The excretion of methylmalonic and formiminoglutamic acids during the induction and remission of vitamin B_{12} deficiency in sheep. *Australian Journal of Biological Sciences* **21**, 789–794.

Gawthorne, J. M. (1970). The effect of cobalt intake on the cobamide and cobinamide composition of the rumen contents and blood plasma of sheep. *Australian Journal of Experimental Biology and Medical Science* **48**, 285–292.

Helmer, L. G. & Bartley, E. E. (1971). Progress in the utilization of urea as a protein replacer for ruminants. A review. *Journal of Dairy Science* **54**, 25–51.

Kovachy, R. J., Copley, S. D. & Allen, R. H. (1983). Recognition, isolation, and characterization of rat liver D-methylmalonyl coenzyme A hydrolase. *Journal of Biological Chemistry* **258**, 11415–11421.

Lough, A. K. & Calder, A. G. (1976). Urinary excretion of methylmalonic and ethylmalonic acids by sheep fed on a barley-rich diet. *Proceedings of the Nutrition Society* **35**, 90A–91A.

McMurray, C. H., Blanchflower, W. J., Rice, D. A. & McLoughlin, M. (1986). Sensitive and specific gas chromatographic method for the determination of methylmalonic acid in the plasma and urine of ruminants. *Journal of Chromatography* **378**, 201–207.

McMurray, C. H., Rice, D. A., McLoughlin, M. & Blanchflower, W. J. (1985). Cobalt deficiency and the potential of using methylmalonic acid as a diagnostic and prognostic indicator. In *Trace Element Metabolism in Man and Animals*, vol. 5, pp. 603–609 [C. F. Mills, I. Bremner and J. K. Chesters, editors]. Slough: Commonwealth Agricultural Bureaux.

Mann, S. O., Wilson, A. B., Barr, M., Lawson, W. J., Duncan, L., Smith, A., Fell, B. F., Walker, H. F. & Macdonald, D. C. (1983). Thiaminase activity in the gut of cobalt-deficient sheep. *Australian Journal of Agricultural Research* **34**, 211–218.

Marston, H. R., Allen, S. H. & Smith, R. M. (1961). Primary metabolic defect supervening on vitamin B_{12} deficiency in sheep. *Nature* **190**, 1086–1091.

Mills, C. F. (1981). Cobalt deficiency and cobalt requirements of ruminants. In *Recent Advances in Animal Nutrition*, pp. 129–141 [W. Haresign, editor]. London: Butterworths.

O'Harte, F. P., Blanchflower, W. J. & Rice, D. A. (1989). Methylmalonic acid as an indicator of vitamin B_{12} deficiency in lambs fed on a cereal-based diet. *Proceedings of the Nutrition Society* **48**, 142A.

Ørskov, E. R. & Grubb, D. A. (1977). The use of whole barley diets fortified with solutions of urea, minerals and vitamins for lambs. *Animal Feed Science and Technology* **2**, 307–314.

Ørskov, E. R., Smart, R. & Mehrez, A. Z. (1974). A method for including urea in whole grains. *Journal of Agricultural Science, Cambridge* **83**, 299–303.

Rice, D. A., McLoughlin, M., Blanchflower, W. J., Goodall, E. A. & McMurray, C. H. (1987). Methylmalonic acid as an indicator of vitamin B_{12} deficiency in grazing sheep. *Veterinary Record* **121**, 472–473.

Rice, D. A., McLoughlin, M., Blanchflower, W. J., McMurray, C. H. & Goodall, E. A. (1989). *Biological Trace Element Research* (In the Press).

Smith, R. M., Osborne-White, W. S. & Russell, G. R. (1967). Metabolism of propionate by sheep liver; pathway of propionate metabolism in aged homogenate and mitochondria. *Biochemical Journal* **104**, 441–449.

Sutherland, R. J. (1980). On the application of serum vitamin B_{12} radioassay to the diagnosis of cobalt deficiency in sheep. *New Zealand Veterinary Journal* **28**, 169–170.

Suttle, N. F. (1986). Problems in the diagnosis and anticipation of trace element deficiencies in grazing livestock. *Veterinary Record* **119**, 148–152.

Underwood, E. J. (1981). Cobalt and nickel. In *The Mineral Nutrition of Livestock*, pp. 113–123 [E. J. Underwood, editor]. Slough: Commonwealth Agricultural Bureaux.

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