Methylmalonyl-CoA mutase (EC 5.4.99.2) and methionine synthetase (EC 2.1.1.13) in the tissues of cobalt–vitamin B₁₂-deficient sheep

BY D. G. KENNEDY¹, A. CANNAVAN¹, A. MOLLOY¹, F. O' HARTE¹,
S. M. TAYLOR², S. KENNEDY³ AND W. J. BLANCHFLOWER¹

Departments of ¹ Biochemistry, ² Haematology and ³ Pathology, Veterinary Research Laboratories, Stoney Road, Belfast BT4 3SD, Northern Ireland, and ⁴ Department of Clinical Medicine, Trinity College, Dublin, Dublin 2, Irish Republic

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The changes in the activities of the two vitamin B₁₂-dependent enzymes methylmalonyl-CoA mutase (EC 5.4.99.2) and methionine synthetase (5-methyltetrahydrofolate–homocysteine methyltransferase, EC 2.1.1.13) are described in two groups of sheep maintained for 20 weeks on either a cobalt-deficient or a Co-sufficient whole-barley diet. At the end of that period, the plasma concentrations of vitamin B₁₂ were depressed and those of methylmalonic acid were raised in the Co-deficient group. During the course of the experiment hepatic holo-mutase activity, measured on biopsy samples, declined in Co-deficient animals with a half-life of 73 d. There was a similar, but slower decline in lymphocyte holo-mutase activity which fell with a half-life of 125 d. At slaughter, there was no difference between Co-sufficient and Co-deficient animals in total mutase activity in liver, kidney, brain and spinal cord. In contrast, the total-synthetase activity of liver and kidney was reduced by 60 and 30% respectively in the Co-deficient animals. There was no change in either group of animals in total-synthetase activity, or in either holo-mutase or holo-synthetase activity, in brain and spinal cord. In the Co-deficient animals, holo-mutase and holo-synthetase activities in liver, the tissue with the greatest activity of both enzymes, fell to 25 and 39% respectively, of that of Co-sufficient animals. The corresponding reductions for kidney were 12 and 51% respectively. These results indicated that activity of both holoenzymes is greatly reduced in Co-deficient sheep.

Cobalt deficiency: Vitamin B₁₂-dependent enzymes: Sheep

There are three main approaches which may be used to assess changes in the activities of the two vitamin B₁₂-dependent enzymes, methylmalonyl-CoA mutase (EC 5.4.99.2) and methionine synthetase (5-methyltetrahydrofolate–homocysteine methyltransferase, EC 2.1.1.13) which accompany the development of cobalt–vitamin B₁₂ deficiency in sheep. First, the activity of the enzymes themselves may be measured. Second, the enzyme activity may be assessed following intravenous (iv) administration of their respective substrates and measurements of the rates of their plasma clearance. Third, the accumulation in plasma and tissue of the enzymes' substrates, their degradation products, or both, may be measured.

Peters & Elliot (1984) showed that there was an increase in the activity of holo-mutase activity in a variety of tissues when Co-deficient sheep were treated with either oral Co or intramuscular hydroxycobalamin. No description of synthetase activity in sheep has been published nor has a description of the changes in the activities of the two vitamin B₁₂-dependent enzymes been made.

Instead, attempts have been made to assess the functional status of the two enzymes by loading with precursors of their respective substrates. For example, Marston et al. (1961) administered propionate by iv injection to sheep and found that it was cleared from the...
plasma more slowly in Co-deficient sheep than in Co-sufficient sheep. These observations were confirmed by Somers (1969) and by Smith & Marston (1971). It was suggested that the impaired metabolism of propionate to succinate which accompanies a decrease in the activity of the mutase enzyme is the 'primary metabolic defect' in ovine Co-deficiency. In addition, Smith & Marston (1971) showed that the increase in plasma glucose concentration which follows the iv administration of propionate occurred more slowly in Co-deficient sheep than in Co-sufficient controls. However, studies from our laboratory (unpublished results) have shown that there is no change in the area under the plasma glucose concentration-time curve following the iv administration of propionate during the first 14 weeks of feeding a Co-deficient diet to sheep. This occurred despite the fact that Co-deficient sheep gained weight at a significantly lower rate than the Co-sufficient controls. Other workers, e.g. Price (1990), have loaded the synthetase enzyme by the iv administration of histidine and measured plasma urocanate concentrations.

The third approach, adopted by many groups, has been to measure either plasma concentrations of methylmalonic acid (MMA), as a diagnostic marker of Co-deficiency (O'Harte et al. 1989), or tissue concentrations of MMA as an alternative indicator of the functional status of the mutase enzyme (Smith et al. 1969). Similarly, formiminoglutamic acid (FIGLU) concentrations in plasma have been measured as markers of the functional status of the synthetase enzyme (Russel et al. 1975). However, unlike MMA, which has now gained widespread acceptance as a diagnostic marker, the use of FIGLU is not common, probably as the result of the unreliability of its assay (Stebbings & Lewis, 1986). Decreased concentrations of S-adenosylmethionine have been recorded in the livers of Co-deficient sheep. This has been regarded also as an indicator of the functional status of the synthetase enzyme (Gawthorne & Smith, 1974).

In the present study, measurements of the activities of both holo-mutase, total-mutase and synthetase were made in selected tissues of Co-deficient sheep. In addition, because of the suggested crucial role of the mutase in the pathogenesis of ovine Co-deficiency, mutase activity was also measured in liver tissues following biopsy and lymphocytes during the course of the study, and MMA concentrations were determined in tissues at slaughter.

**EXPERIMENTAL**

*Animals, experimental design, diet and sampling*

Eight 9-month-old Suffolk Cross lambs were randomly allocated to two groups. The groups were separately housed and allowed access to water and feed ad lib. The duration of the experiment was 20 weeks.

The groups were fed on a whole barley-based diet which was either Co-sufficient (1000 µg/kg) or Co-deficient (4-2 µg/kg) as described earlier (O'Harte et al. 1989).

At intervals during the course of the experiment heparinized blood samples (20 ml) were collected. Plasma was prepared and stored at $-20^\circ$ for the subsequent determination of vitamin $B_{12}$ and MMA. At the same time, oxalate–fluoride blood samples (5 ml) were collected for the immediate determination of plasma glucose. Heparinized blood samples (50 ml) were collected at intervals. From these, lymphocytes were purified as described later (p. 723) and stored at $-70^\circ$ before assay for mutase activity.

On three occasions during the experiment, liver biopsies (about 1 g) were taken under general anaesthesia. The liver samples were stored under liquid nitrogen before assay for mutase activity. On one occasion, one of the Co-sufficient animals failed to recover from the anaesthesia and the experiment continued using only three control animals.

On termination of the experiment at day 140, tissue samples (about 10 g) and cerebrospinal fluid (CSF, about 1 ml) were taken at autopsy. The tissue samples were stored
under liquid N₂ before assay for enzyme activity and CSF held at −20° before assay for MMA.

**Routine assays**

True plasma vitamin B₁₂ was measured using the Becton Dickinson radioassay kit (Becton Dickinson Immunodiagnostics, New York, USA). Plasma MMA was measured using a capillary gas–liquid chromatographic assay developed in our laboratory (McMurray et al. 1986). Plasma glucose was measured on a Hitachi 705 autoanalyser using a glucose test kit (Randox Laboratories, Northern Ireland). Tissue concentrations of vitamin B₁₂ were measured using the radioassay kit following extraction of tissue homogenates as prepared later for holo-mutase activity with a citrate–phosphate buffer as described by Kennedy et al. (1990). Tissue homogenates were directly substituted for serum using this method. Tissue concentrations of MMA were measured using the homogenates prepared later for holo-mutase activity.

**Enzyme assays**

(a) Mutase: preparation of lymphocytes. Blood (60 ml) in eight portions was centrifuged with Ficoll-Paque (5.6 ml per portion) in 15 ml plastic centrifuge tubes at 1400 g for 30 min at 4°. The lymphocyte layers were combined in a tapered 15 ml centrifuge tube and pelleted by centrifugation at 1000 g for 30 min at 4°. The supernatant fractions were aspirated and contaminating erythrocytes flash-lysed by resuspending the pellet in distilled water (4 ml) for 60 s at room temperature. This was followed by the addition of sodium chloride (27 g/l; 2 ml) to restore isotonicity. This suspension was then centrifuged at 1000 g for 30 min at 4° to pellet the cells. Following aspiration of the supernatant fraction, the cell pellets were stored at −70° before assay.

Immediately before assay, the cells were thawed and resuspended in 0.014 M-sodium phosphate buffer, pH 7.0, containing 2.8 mM-reduced glutathione (800 μl), and sonicated using an MSE sonicator (six 10 s bursts and placed in ice between each burst).

Preparation of tissues. Frozen tissue samples were thawed and approximately 0.5 g portions were homogenized for 60 s in 10 vol. 0.014 M-sodium phosphate buffer, pH 7.0, containing 2.8 mM-reduced glutathione, using an MSE homogenizer. The homogenates were cooled to 4° and centrifuged at 10000 g for 15 min at 4°. Supernatant fractions, diluted 1:10 (v/v) with the phosphate buffer were assayed for mutase activity.

Holo-mutase activity. Holo-mutase (enzyme containing bound 5'-deoxyadenosyl cobalamin) activity was measured by a modification of the method of Kolhouse & Allen (1977). Sample (100 μl) along with 0.1 M-Tris-hydrochloride buffer, pH 8.5, containing 2.8 mM-reduced glutathione (100 μl) was added to 3 ml glass tubes which had ground-glass stoppers. Then, 18 μM-DL-2-[methyl-¹⁴C]methylmalonyl CoA (100 μl, equivalent to 0.1 μCi and 1.8 nmol, obtained from New England Nuclear) and 600 μM-DL-2-methylmalonyl CoA (300 μl, equivalent to 180 nmol), both in Tris-hydrochloride buffer, were added. The mixtures were incubated for 60 min (lymphocytes) or 15 min (tissues) at 37°. The reaction was stopped by the addition of 2 M-perchloric acid (100 μl) and heating to 100° for 3 min. The samples were then centrifuged at 1500 g for 10 min at 4°. Portions (450 μl) of supernatant fraction were transferred to clean tubes and 2 M-perchloric acid (50 μl) and potassium permanganate (40 g/l, 500 μl) added. The tubes were heated in a boiling water bath for 10 min, then centrifuged at 1500 g for 15 min at 4°. Portions (700 μl) of supernatant fractions were transferred to scintillation vials containing 250 μl propionic acid and the samples dried overnight at 90°. Distilled water (1 ml) and scintillation fluid (10 ml) were added and radioactivity determined using a liquid scintillation spectrometer. Assays were carried out in triplicate. Total count tubes (no permanganate) and blank tubes (no sample)
Fig. 1. Changes in plasma concentrations of vitamin B₁₂ (■) and methylmalonic acid (MMA) (○) in lambs fed on a cobalt-deficient whole-barley diet. Values are means, with their standard errors represented by vertical bars. For details of dietary treatment, see p. 722.

were included in every run. Protein concentrations in the samples were determined using the method of Lowry et al. (1951) and activity expressed as units (nmol succinate formed/h) per mg protein.

Total-mutase activity. This was measured as described previously, with the substitution of 0.3 mM-5′-deoxyadenosyl cobalamin (100 μl) in Tris-hydrochloride buffer for Tris-hydrochloride buffer. The assay was carried out under dim red lighting from this addition until the addition of perchloric acid.

(b) Synthetase activity. This was measured using the method of Keating et al. (1985) modified to enable determinations of both holo- and total-synthetase activity to be made by the omission and inclusion of cyanocobalamin respectively.

Statistical analysis

Values are presented as means with their standard errors. Statistical tests are two-tailed equal variance t tests, unless stated otherwise.

RESULTS

Plasma vitamin B₁₂ and MMA

The mean plasma concentrations of vitamin B₁₂ and MMA did not change significantly during the study in the Co-sufficient animals and were 1590 (SE 63) pmol/l (n 34) and 0.70 (SE 0.06) μmol/l (n 34) respectively. Fig. 1 shows the mean plasma concentrations of vitamin B₁₂ and MMA in animals fed on the Co-deficient diet. The mean plasma vitamin B₁₂ concentrations in these animals were significantly lower than those in the Co-sufficient animals from day 15 onwards (P < 0.01) and fell below 220 pmol/l by about 53 d. The mean plasma concentration of MMA in the animals fed on the Co-deficient diet rose gradually up to day 112, moving above 10 μmol/l by day 96. Therefore the rate of increase in the concentration of plasma MMA rose sharply. At day 140, the mean plasma MMA
Table 1. *Tissue vitamin $B_{12}$ and methylmalonic acid (MMA) concentrations in cobalt-sufficient and Co-deficient sheep*.

(The tissue concentrations of vitamin $B_{12}$ were determined using tissue homogenates prepared freshly and extracted by boiling in a citrate phosphate buffer. The tissue concentrations of MMA were determined using tissue homogenates which had been prepared for determination of holo-mutase activity. Values are means with their standard errors. Vitamin $B_{12}$ values were analysed using a one-tailed $t$ test, applying equal or unequal variance tests, as appropriate. MMA values were analysed using Wilcoxon’s ranked sum test)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sufficient Mean (pmol/g wet wt)</th>
<th>Sufficient SE</th>
<th>Deficient Mean (pmol/g wet wt)</th>
<th>Deficient SE</th>
<th>Statistical significance of difference</th>
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</thead>
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<tr>
<td>Liver</td>
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<tr>
<td>Kidney</td>
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<td>83 7</td>
<td>83 7</td>
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<tr>
<td>Brain</td>
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<td>55 5</td>
<td>55 5</td>
<td>P &lt; 0.05</td>
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</tr>
<tr>
<td>CSF</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
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MMA concentration (nmol/g wet wt)

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<tr>
<th>Tissue</th>
<th>Sufficient Mean (nmol/g wet wt)</th>
<th>Sufficient SE</th>
<th>Deficient Mean (nmol/g wet wt)</th>
<th>Deficient SE</th>
<th>Statistical significance of difference</th>
</tr>
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<tr>
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<td>333 276</td>
<td>333 276</td>
<td>P &lt; 0.005</td>
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<tr>
<td>Kidney</td>
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<td>185 136</td>
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<td>CSF</td>
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<td>2.2 0.9</td>
<td>NS</td>
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</tr>
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</table>

ND, not determined; NS, not significant; CSF, cerebrospinal fluid.

* For details of dietary treatments, see p. 722.

concentration was 140 μmol/l (this point has been omitted from Fig. 1 for clarity). Although the between-animal variation was high, the plasma concentrations of MMA in the animals fed on the Co-deficient diet were significantly higher than those in the control group from day 81 onwards ($P < 0.05$, Wilcoxon’s rank sum test).

**Tissue concentrations of vitamin $B_{12}$ and MMA**

Table 1 shows that the mean concentration of vitamin $B_{12}$ was lower in the deficient group than the control group in all the tissues examined. However, the difference in vitamin $B_{12}$ content of spinal cord was not statistically significant and that in kidney narrowly failed to reach statistical significance. The latter case was despite the fact that the mean value for the deficient animals was 17% of the control values, and reflects the large variation in the kidney vitamin $B_{12}$ concentration in the Co-sufficient animals (coefficient of variation 50%). The mean concentration of MMA was higher in the tissues of the Co-deficient animals than those of Co-sufficient animals but only the MMA concentrations of liver and kidney were statistically significantly increased (by 50- and 120-fold respectively).

**Plasma glucose**

At no time during the course of the experiment were the mean plasma concentrations of glucose in animals fed on the Co-sufficient diet significantly different from those fed on the Co-deficient diet.

**Lymphocyte mutase activity**

Fig. 2 shows the holo-mutase activity in jugular blood lymphocytes during the course of the experiment. The mean holo-mutase activity in lymphocytes from animals fed on the Co-sufficient diet fell from 9.1 (SE 0.9) units/mg protein ($n$ 4) to 7.5 (SE 0.2) units/mg protein ($n$ 3) after about day 96, remaining at about that level thereafter. This decrease was not statistically significant. The holo-mutase activity in the lymphocytes of the animals fed on the Co-deficient diet declined monoexponentially ($r^2$ 0.960) with a half-life of 125 d from 10.1 (SE 0.9) units/mg protein to 4.5 (SE 0.75) units/mg protein and was significantly lower.
Fig. 2. Holo-methylmalonyl-CoA mutase (holo-mutase) (EC 5.4.99.2) activity in lymphocytes from sheep fed on either a cobalt-sufficient (■) or Co-deficient (○) whole-barley diet. Values are means with their standard errors represented by vertical bars. For details of dietary treatments, see p. 722.

than the initial value from day 53 onwards ($P < 0.01$). The difference in the holo-mutase activity in the Co-sufficient and Co-deficient groups was significant at 125 and 133 d ($P < 0.05$ and $P < 0.01$ respectively).

The total-mutase activity recorded in the lymphocytes from both groups did not change during the course of the experiment and had a mean value of 55.1 (SE 18.4) units/mg protein ($n = 16$).

The percentage saturation of the mutase with cobalamin cofactor (that is, the percentage contribution of holo-mutase activity to total-mutase activity) was approximately 19 (SE 7)% in the control animals. In the Co-deficient animals there was a significant decrease in the percentage saturation of the enzyme from 22 (SE 3)% to 9 (SE 2)% at day 133 ($P < 0.01$).

Liver mutase activity

Fig. 3 shows the decline in hepatic holo-mutase activity in animals fed on the Co-deficient diet. The holo-mutase activity declined monoexponentially ($r^2 = 0.997$) with a half-life of 73 d from an initial value of 415 (SE 40) units/mg protein to 108 (SE 20) units/mg protein (26% of that at day 0) at day 140. This difference was highly significant ($P < 0.001$). There was no change in the hepatic holo-mutase activity of the Co-sufficient animals during the course of the study (mean value 452 (SE 45) units/mg protein).

The total-mutase activity did not change during the course of the experiment ($P > 0.1$) and was 1556 (SE 117) units/mg protein ($n = 16$). The percentage saturation of the hepatic mutase fell significantly from 27 (SE 3)% at day 0 to 7 (SE 1)% at day 140 ($P < 0.001$).

Tissue mutase activity

Fig. 4 shows the holo-mutase activity in liver, kidney, brain and spinal cord of animals of each group at slaughter. Liver mutase activity in deficient animals was 25% of that in controls ($P < 0.05$). Similarly, kidney mutase activity in deficient animals was 12% of that in controls ($P < 0.001$). However, the holo-mutase activity of neither brain nor spinal cord was affected by the Co-deficiency.
Fig. 3. Hepatic holo-methylmalonyl-CoA mutase (holo-mutase) (EC 5.4.99.2) activity in sheep fed on a cobalt-deficient whole-barley diet. Values are means, with their standard errors represented by vertical bars. For details of dietary treatment, see p. 722.

Fig. 4. Holo-methylmalonyl-CoA mutase (holo-mutase) (EC 5.4.99.2) activity in the tissues of cobalt-sufficient (■) and Co-deficient sheep (□). Values are means with their standard errors represented by vertical bars. For details of dietary treatments, see p. 722.
The total-methionase activity of all four tissues was unaffected by Co-deficiency. The overall mean values were (units/mg protein): liver 1889 (SE 157), kidney 223 (SE 42), brain 164 (SE 14), spinal cord 188 (SE 25).

**Tissue synthetase activity**

Fig. 5 shows the total-synthetase activity in liver, kidney, brain and spinal cord of animals fed on the Co-sufficient and Co-deficient diets at slaughter. Total-synthetase activity in liver of deficient animals fell to 40% (P < 0.01) of control values and in kidney to 70% (P < 0.05) of control values. Co-deficiency did not affect total-synthetase activity in either brain or spinal cord.

Fig. 6 shows the holo-synthetase activity in liver, kidney and brain of animals fed on the Co-sufficient and Co-deficient diets at slaughter. Liver holo-synthetase activity in liver of Co-deficient animals was 39% of that in Co-sufficient controls (P < 0.001). Kidney holo-synthetase activity in deficient animals was 51% of that in controls (P < 0.05). However, the holo-synthetase activity of brain was not affected by Co-deficiency.

**DISCUSSION**

Fig. 1 showed that the plasma concentration of vitamin B\textsubscript{12} fell below the lower limit of normality (220 pmol/l) by day 53 and that MMA concentrations in plasma rose above the upper limit of normality (10 μmol/l) within 95 d in animals fed on the Co-deficient diet. In a previous study from our laboratory (O’Harte et al. 1989) the corresponding values were 35 and 63 d respectively. The longer time interval required to develop vitamin B\textsubscript{12} deficiency in the present study was not related to the Co concentration in the Co-deficient ration (4.2 μg/kg in both studies). However, the animals used in the present study were 36 weeks old, compared with the 10-week-old animals used by O’Harte et al. (1989). Thus it is speculated that the larger animals may have had larger reserves of vitamin B\textsubscript{12} and may have been growing more slowly with consequently a lower Co requirement.

The values presented in Figs. 2, 3 and 4 show that holo-mutase activities in different tissues of Co-deficient sheep vary widely. Kidney seems to be particularly sensitive to
cobalamin deficiency. At day 140, kidney holo-mutase activity in Co-deficient animals was reduced to less than 15% of that in Co-sufficient animals (Fig. 4). The corresponding values for liver and lymphocytes (at day 133) were 25 and 47% respectively (Figs. 4 and 3 respectively). The findings in brain and spinal cord are in marked contrast. In both tissues the holo-mutase activity in the Co-deficient animals was similar to that in the Co-sufficient controls. It is not clear why there should be such a dramatic difference between tissues. It is possible that the mutases vary in their binding affinity for 5'-deoxyadenosyl cobalamin. Another possibility is that depletion of holo-mutase activity develops during normal enzyme turnover under conditions when little or no coenzyme $B_{12}$ is available to form the holo-enzyme. Hence, different rates of enzyme turnover in tissues would lead to dissimilar responses of holo-mutase activity to vitamin $B_{12}$ deficiency.

Peters & Elliot (1984) presented results similar to those obtained in the present study, despite having a very different protocol. They fed sheep on a diet containing 60 $\mu$g Co/kg (approximately fourteen times more than that in the present study) for 7 months. In addition, they did not have a group of Co-sufficient controls. Instead, they split their experimental animals into three groups after 7 months, leaving one group Co-deficient and repleting the other two groups using either oral Co or intramuscular hydroxocobalamin on alternate days for 9 weeks. Hence, their repleted animals were not true Co-sufficient controls. Nonetheless, their findings compare favourably with those obtained in the present study. The major difference was that in brain as well as in liver and kidney, they observed increases in holo-mutase activity on supplementation with Co or hydroxocobalamin. Whether this reflects a more severe deficiency in their animals or their choice of a control group is not clear. The percentage contributions of holo-mutase activity in unsupplemented animals to those in their Co-supplemented animals for kidney, liver and brain were 12, 25 and 66 respectively, which are very similar to those obtained in the present study. Their finding of changes in brain holo-mutase activity may reflect the fact that their Co-deficient animals were given their diet for a total of 37 weeks in comparison with the 20 weeks in the present study.
The relationship between holo-mutase activity and plasma MMA concentration is quite complex. Lymphocyte holo-mutase activity in animals fed on the Co-deficient diet on day 53 was 66% of that at day 0 (Fig. 2). Hepatic holo-mutase activity was subject to a similar decline (66% of values at day 0 by day 49, Fig. 3). However, the plasma concentration of MMA was essentially unchanged at day 53 having risen from 0.81 (±0.18) to 2.85 (±1.49) μmol/l. Thereafter, a relatively slight change in holo-mutase activity in lymphocytes was reflected in massive changes in plasma MMA concentrations. Between days 96 and 133 holo-mutase activity in lymphocytes from Co-deficient sheep fell from 58 to 47% of their values at day 0. The corresponding changes in plasma MMA concentrations were from 15 to 65 μmol/l, both values being in excess of the upper limit of normality (10 μmol/l for barley-fed Co-sufficient sheep).

In all the tissues studied, the vitamin B₁₂ concentration was decreased as a result of Co-depletion. However, the decrease failed to reach statistical significance in the spinal cord and narrowly (P = 0.053) failed in the kidney (Table 1). Liver and kidney, both of which had a decreased holo-mutase activity also had increased concentrations of MMA (Table 1). The concentration of MMA in the liver in the present study (333 nmol/g) was higher than that reported by Smith et al. (1969) (64–126 nmol/g) suggesting that a more severe deficiency had been induced in the present study. Smith et al. (1969) did not report values of MMA in other tissues. Brain, spinal cord and CSF concentrations of MMA in deficient animals were not significantly different from Co-sufficient controls, although in all three cases the mean MMA concentrations were higher in the Co-deficient animals.

In none of the tissues studied was there any significant increase in total-mutase activity as a result of vitamin B₁₂ deficiency. This contrasts with Cardinale et al. (1969) who reported a significantly increased total-mutase activity in rat liver. In Co-sufficient animals the percentage contribution of holo-mutase to total-mutase (i.e. percentage saturation of total-mutase with coenzyme B₁₂) averaged 24%. It might be suggested that the higher the percentage saturation of the mutase in a particular tissue, the more resistant that tissue will be to cobalamin deficiency, where resistance is defined by residual holo-mutase activity and tissue MMA concentration. This is not supported by the evidence since brain and spinal cord, which had the highest (33%) and lowest (15%) percentage saturation, showed no changes in either holo-mutase activity or tissue MMA concentration in Co-deficiency.

Despite recording large decreases in hepatic holo-mutase activity, the changes in plasma concentrations of glucose were not significant. This contrasts with the findings of MacPherson et al. (1976), who showed decreased plasma concentrations of glucose in Co-deficient lambs. However, those workers noted that the changes in plasma glucose concentration became consistently abnormal after 34 weeks of feeding their experimental diet. Given the apparent importance of propionate in gluconeogenesis in sheep and the severity of the reduction of the activity of hepatic holo-mutase (Fig. 4), it is somewhat surprising that an alteration in plasma glucose is apparently such a late manifestation of Co-deficiency in sheep. Glucose synthesis arising from protein catabolism probably accounts for the late onset of changes in plasma glucose concentrations.

The finding that total-synthetase activity is decreased in the liver and kidneys of Co-deficient sheep is very surprising. When a particular enzyme is subjected to metabolic stress by substrate deprivation, the organism frequently responds to up-regulating either the activity or the amount of the enzyme in an attempt to ‘mop up’ whatever substrate is available. Examples of enzymes which have been reported to be subject to this phenomenon include the Na,K-ATPase (EC 3.6.1.3; Kennedy et al. 1986), the glucose transport system (Martineau et al. 1972), the neutral amino acid transport system (Heaton & Gelehrter, 1977) and even the mutase enzyme (Cardinale et al. 1969). Down-regulation of enzyme activity in response to this kind of metabolic stress is, however, highly unusual. There is no
obvious explanation for these findings, but it may be speculated that synthesis of the synthetase requires the presence or participation of vitamin $B_{12}$. Hence in tissues which become vitamin $B_{12}$ deficient, de novo biosynthesis of the enzyme is inhibited and total-enzyme activities decline as a result of normal enzyme turnover. Hence, total-synthetase activities in brain and spinal cord, where vitamin $B_{12}$ concentrations are not significantly reduced, are unchanged.

Holo-synthetase activity from the three tissues for which values are available are more in line with the equivalent results for the mutase enzyme. Both liver and kidney holo-synthetase activities were depressed while that in the brain was unaffected by Co-deficiency.

Two interesting observations may be made from the findings presented here. First, the decline in the activities of holo-mutase and holo-synthetase was broadly similar in all tissues studied, although the fall in holo-mutase activity in kidney, to 12% of controls, was much greater than that of holo-synthetase activity which fell to 51% of controls. This suggests that describing the inhibition of mutase activity as the primary metabolic defect in Co-deficient sheep may be, at best, simplistic. Clearly, a rigorous examination of the metabolic consequences of inhibition of both enzymes in Co-deficiency in sheep is indicated. Second, holo-enzyme activities in different tissues decline at different rates. In particular brain and spinal cord seem fairly resistant to vitamin $B_{12}$ deficiency. However, the findings show that even in these tissues, vitamin $B_{12}$ concentrations are lower than in control animals, suggesting that neural tissue may eventually become affected by a functional vitamin $B_{12}$ deficiency. These findings may, at least in part, offer an explanation as to why vitamin $B_{12}$-deficient sheep do not apparently develop any neurological dysfunction such as ataxia which has been reported in humans (Pant et al. 1968), monkeys (Scott et al. 1981), fruit bats (Metz & van der Westhuyzen, 1987) and nitrous oxide-treated pigs (Weir et al. 1988).

Although it has been believed for some time that a dysfunction of the mutase enzyme causes dysmyelination in humans (Frenkel, 1973; Kishimoto et al. 1973) mediated by an accumulation of odd number/branched-chain fatty acids in the brain, there is now increasing evidence that a disturbance of synthetase activity and methylation reactions is responsible (Scott et al. 1981; Small et al. 1981; Weir et al. 1988). However, until the studies outlined later are complete, it will not be possible to speculate further on the pathogenesis of dysmyelination since neither mutase nor synthetase activities were affected in neural tissue in the present experiment. The present study was directed principally towards determining the activities of the two enzymes, but with particular emphasis on the mutase enzyme. A follow-up study is currently in progress which will redress the balance by measuring enzyme activity, MMA, S-adenosyl methionine and S-adenosyl homocysteine concentrations, odd-number branched-chain fatty acids and phosphatidyl choline: phosphatidyl ethanolamine ratios together with a full histopathological examination in severely Co-deficient animals.

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REFERENCES


