Cobalt–vitamin B_{12} deficiency causes accumulation of odd-numbered, branched-chain fatty acids in the tissues of sheep

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Nine 5-month-old lambs were randomly allocated to two groups and were fed on either a Co-deficient whole-barley diet (*n* 5), or the same diet supplemented with Co (*n* 4). The lambs were fed on their respective diets for 28 weeks. Plasma vitamin B_{12} concentrations fell below the lower limit of normality after 6 weeks, and plasma methylmalonic acid (MMA) concentrations rose above the upper limit of normality after 10 weeks. However, plasma MMA concentrations fell to near normal levels towards the end of the experiment suggesting that diagnosis of more severe Co deficiency based on determination of plasma MMA concentrations may be of limited value. Analysis of tissue samples collected at slaughter revealed a marked reduction in the vitamin B_{12} concentration and the activity of methylmalonyl-CoA mutase (*EC* 5.4.99.2) in the tissues taken from the Co-deficient sheep, by comparison with the controls. Although tissue concentrations of MMA in the Co-deficient animals were not significantly different from those of the controls, we did detect increased concentrations of branched-chain fatty acids. This suggested that misincorporation of MMA, but not propionic acid, into fatty acids had occurred. The Co-deficient lambs did not develop any neurological signs, suggesting that accumulation of branched-chain fatty acids may not be involved in the development of neurological lesions.

Vitamin B_{12}: Methylmalonic acid: Branched-chain fatty acids: Sheep

Co, an essential trace element for ruminants, is not known to have any biological function *per se*. However, it is required for the rumen synthesis of vitamin B_{12}. The natural diet of herbivores is devoid of vitamin B_{12}, so ruminants are entirely dependent on the maintenance of a steady supply of Co for the microbial synthesis of this vitamin in the rumen. Consequently, sheep fed on a Co-deficient diet (providing $< 70 \mu g$ Co/kg diet) rapidly develop vitamin B_{12} deficiency, which is characterized clinically by ill thrift and appetite reduction (Marston, 1970).

Vitamin B_{12} acts as the cofactor for two enzymes, namely methylmalonyl-CoA mutase (*EC* 5.4.99.2; MM-CoA mutase) and methionine synthase (*EC* 2.1.1.13). The enzyme MM-CoA mutase catalyses the conversion of L-methylmalonyl-CoA (MM-CoA) to succinyl-CoA and is of central importance in energy metabolism in sheep. Sheep synthesize up to 40 % of their daily glucose requirement via gluconeogenesis in the liver (Bergman *et al.* 1966). The main substrate for this is propionate, the only gluconeogenic volatile fatty acid produced during normal rumen fermentation. In ovine vitamin B_{12} deficiency, metabolism of propionate to succinate is impaired as a result of the reduced activity of the mutase enzyme (Kennedy *et al.* 1991*a*). As a result, MM-CoA and propionyl-CoA are postulated to accumulate in tissues. The former compound may be degraded to methylmalonic acid (MMA; Kovachy *et al.* 1983) which, it has been demonstrated,

accumulates in tissue and plasma (O'Harte *et al.* 1989) and is excreted in urine (Millar & Lorentz, 1979).

Fatty acid synthesis normally proceeds by the sequential condensation of malonyl-CoA units to acetyl-CoA, to form fatty acids up to C_{18}. However, propionyl-CoA can substitute for acetyl-CoA (Frenkel *et al.* 1973) and MM-CoA for malonyl-CoA (Scaife *et al.* 1978). Thus, organic acidaemias, which lead to tissue accumulation of propionyl-CoA and MM-CoA, can cause an accumulation of odd-numbered straight- and branched-chain fatty acids respectively.

It has been suggested that the odd-numbered straight- and branched-chain fatty acids thus formed may be functionally impaired and that excessive accumulation of these compounds may be responsible for some of the neurological complications associated with human vitamin B_{12} deficiency (Frenkel, 1973; Kishimoto *et al.* 1973; Ramsey *et al.* 1977). To date, only one study has attempted to quantify the accumulation of these fatty acids in the tissues of Co-deficient sheep (Duncan *et al.* 1981). However, that study measured the concentrations of these compounds in the tissues of neonatal lambs born to Co-deficient ewes whereas, characteristically, Co deficiency is a disease of growing lambs. In addition, this earlier study did not measure either MM-CoA mutase activity or MMA concentration in tissues, nor did it estimate synthesis of odd-numbered straight- and branched-chain fatty acids in neural tissues. The present study describes accumulation of odd-numbered straight- and branched-chain fatty acids in a range of tissues from both moderately and severely Co-deficient lambs, and relates this increase to the tissue concentrations and activities of MMA and MM-CoA mutase respectively.

MATERIALS AND METHODS
Experimental diet
Co-deficient barley was purchased from a farm in Northern Ireland. The barley was supplemented with urea, DL-α-tocopheryl acetate, cholecalciferol, retinoyl palmitate and minerals as described earlier (O'Harte *et al.* 1989). The Co contents of the deficient and sufficient diets, as measured by graphite furnace atomic absorption spectrophotometry (Blanchflower *et al.* 1990), were 4·5 and 1000 μg/kg respectively.

Animal studies
(a) *20 week depletion.* Only the previously unpublished values for tissue concentrations of odd-numbered branched- and straight-chain fatty acids are presented in the present study. Details concerning the animals and other relevant biochemical effects of the 20-week period of Co depletion have all been described in an earlier publication (Kennedy *et al.* 1990*a*).

(b) *28 week depletion.* Nine 5-month-old Suffolk Cross lambs, bred at the laboratory, were randomly allocated to two groups. One group of five animals was fed on the Co-deficient diet while the other group of four animals was fed on the same diet supplemented with Co to a level of 1000 μg/g. The groups were separately housed in indoor pens with wire flooring. The animals were fed on either the Co-deficient or the Co-sufficient diet *ad lib.* for 28 weeks. Mean voluntary feed intake (VFI) and live-weight gain were measured at weekly intervals during the study. Plasma samples were obtained at 14 d intervals by jugular venepuncture and were assayed for vitamin B_{12} and MMA concentrations.

At the end of the experiment the animals were killed by intravenous pentobarbitone overdose. Tissue samples were taken and stored under liquid N_2 before assay. All data presented here on VFI, live-weight gain, plasma and tissue concentrations of vitamin B_{12} and MMA and activity of MM-CoA mutase relate to these animals only.

Analytical procedures

Plasma concentrations of 'true' vitamin B_{12} were determined using a radioimmunoassay kit (Becton Dickinson Immunodiagnostics, Orangeberg, NY, USA). Tissue concentrations of vitamin B_{12} were measured as described previously (Kennedy *et al.* 1990*b*). Total and holo MM-CoA mutase activities were measured radio-enzymically as described by Kennedy *et al.* (1990*a*). Apo-MM-CoA mutase activity was then calculated by subtraction of holo enzyme activity from total enzyme activity. Plasma and tissue concentrations of MMA were measured using capillary GLC, as described previously by McMurray *et al.* (1986) and by Kennedy *et al.* (1990*a*) respectively.

Determination of tissue odd-numbered branched- and straight-chain fatty acid concentrations

Minced tissue (1 g) was extracted as described by Folch *et al.* (1957) to give a solution of 10 g lipid/l chloroform–methanol (2:1, v/v). A portion (500 μl) of each extract, along with a portion (50 μl) of the internal standard, heneicosanoic acid (1 g/l), was dried under N_2 at 60°. BF_3 in methanol (500 μl) was added to each tube which were then capped and heated to 70° in a water-bath for 30 min. After cooling, water (1 ml) was added and the mixture extracted with hexane (400 μl). A portion of the hexane solution was transferred to autosampler vials for analysis by GLC–MS. The GLC–MS system consisted of a Hewlett Packard 5995 quadrupole GLC–MS interfaced to a Hewlett Packard Chemstation data system. The outlet of the CP-Sil 88 column (25 m × 0·2 mm) was directly inserted into the ion source. The temperatures of the transfer line, source and analyser were 220, 150 and 150° respectively. A Hewlett Packard 7671A autosampler was used to inject portions (2 μl) of the derivatized sample extracts into the injector port of the GLC, which was maintained at 240°. The temperature gradient used was 150 to 220° at 5° per min, with a sample split ratio of 20:1. Four ions were monitored, at m/z 256·4, 284·5, 312·5 and 340·5 for C_{15}, C_{17}, C_{19} and C_{21} respectively. Peaks were compared with standards (1 g/l) of each fatty acid similarly treated. Branch-chain fatty acids elute from the column just before their straight-chain equivalents.

Statistical analyses

Single classification ANOVA was used for analysis of plasma vitamin B_{12} and MMA concentrations and for VFI and live-weight gain. Student's two-tailed *t*-test, using equal or unequal variance as appropriate, was used in all other cases. Differences between means were considered significant at $P < 0.05$. Data are presented as means with their standard errors.

RESULTS

Plasma concentrations of vitamin B_{12} and MMA (28 week depletion)

The plasma vitamin B_{12} concentration of the animals fed on the Co-deficient diet was significantly lower than that of the Co-sufficient controls ($P < 0.05$) after 2 weeks (Fig. 1). It then fell below the lower limit of normality (220 pmol/l; O'Harte *et al.* 1989) by 6 weeks and remained below that level for the remainder of the study. The mean plasma vitamin B_{12} concentration of the control animals remained in excess of 1600 pmol/l throughout the study (values not shown).

The mean plasma MMA concentration of the animals fed on the Co-deficient diet rose above the upper limit of normality for barley-fed sheep (10 μmol/l; O'Harte *et al.* 1989; Kennedy *et al.* 1990*b*, 1992*b*) after 10 weeks, and was significantly higher than that in the
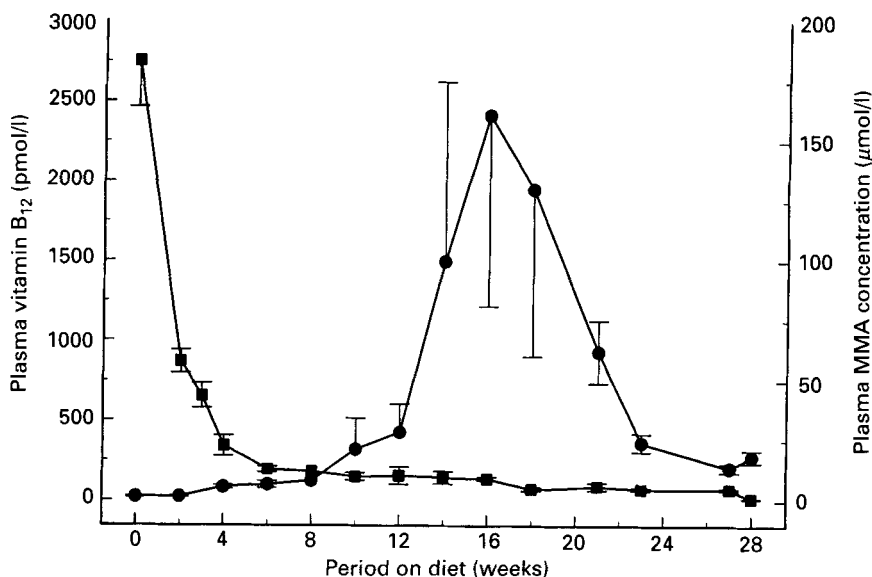
Fig. 1. Changes in the concentrations of vitamin B_{12}, measured by radioimmunoassay (■), and methylmalonic acid (MMA), measured by capillary GLC (●), in the plasma of five sheep fed on a cobalt-deficient diet (4·5 ng Co/g) for 28 weeks. Values are means with their standard errors represented by vertical bars. For details of animals and procedures, see pp. 68–69.

control animals from week 14 ($P < 0.05$). The levels continued to rise until week 16, when they peaked with a mean MMA concentration of 160 μmol/l. Thereafter the mean levels fell steadily, ceasing to be significantly different from those for the control animals from 20 weeks until the end of the study (Fig. 1). The mean plasma MMA concentration in the control animals did not change during the course of the study and was 2·4 μmol/l (values not shown).

VFI and live-weight gain (28 week depletion)

The live weight of the animals fed on the Co-sufficient diet increased steadily during the study (Fig. 2a). For the first 10 weeks the mean live-weight gain was 210 (SE 10) g/d. In contrast, the live weights of the animals fed on the Co-deficient diet increased at a slower rate, equivalent to 122 (SE 7) g/d over the same period. Thereafter, their live weight did not change significantly until 19 weeks, when they began to lose weight rapidly. At 28 weeks they were only 83 % of their live weight at week 0. The differences in weight gain became significant ($P < 0.05$) from week 4 to the end of the study. The mean four-weekly VFI for the animals fed on the Co-sufficient diet did not change significantly during the course of the study (Fig. 2b) and was 8·2 (SE 0·2) kg/sheep. However, the mean weekly VFI for the animals fed on the Co-deficient diet was significantly lower than that for the Co-sufficient controls throughout the study ($P < 0.05$). By the last week of the study the VFI for the Co-deficient animals was 22 % of that for the control animals.

Vitamin B_{12} and MMA concentrations and MM-CoA mutase activity in tissues (28 week depletion)

The vitamin B_{12} concentrations in the tissues of the Co-deficient animals were significantly lower than that for the controls (Table 1). However, there was no difference in tissue MMA concentrations between the control and Co-deficient groups (Table 1). The activity of MM-
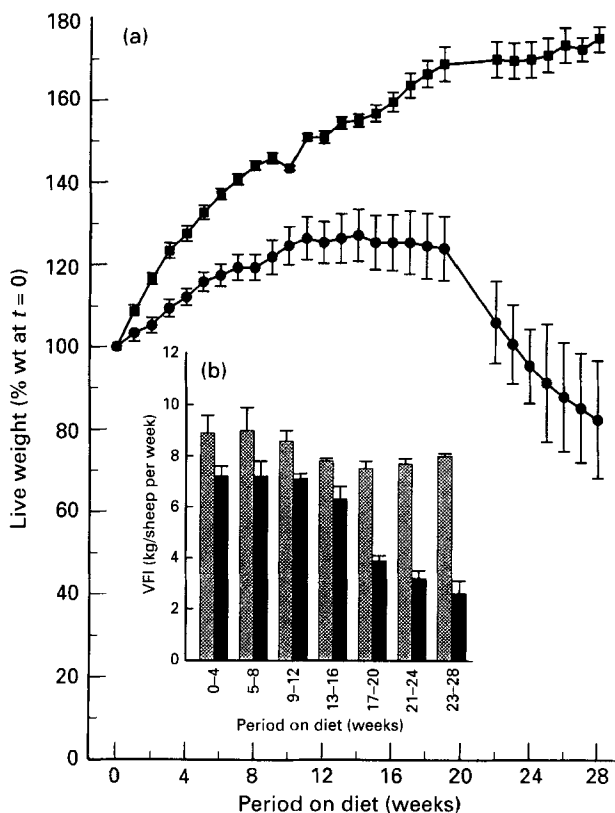
Fig. 2. Live-weight gain and voluntary feed intake (VFI). (a) Weekly live-weight gains were measured in sheep fed on either a cobalt-sufficient diet (n 4; 1000 ng Co/g; (■)) or a Co-deficient diet (n 5; 4·5 ng Co/g; (●)) for 28 weeks. (b) Mean weekly VFI were measured at four-weekly intervals in the same sheep (Co-sufficient (▨), Co-deficient (■)). Values are means with their standard errors represented by vertical bars. For details of animals and procedures, see pp. 68–69.

Table 1. *Vitamin B_{12} and methylmalonic acid concentrations in the tissues of sheep deprived on dietary cobalt for 28 weeks†*

(Mean values with their standard errors)

| | Vitamin B_{12} (pmol/g wet wt) | | | | Methylmalonic acid (nmol/g wet wt) | | | |
| | +Co (n 4) | | −Co (n 5) | | +Co (n 4) | | −Co (n 5) | |
Tissue	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Liver	395·5	9·7	20·0***	4·2	5·1	0·4	23·3	9·2
Kidney	349·0	23·0	26·5***	4·0	4·7	0·7	7·0	2·9
Brain	87·7	1·0	31·3**	3·1	2·7	1·6	6·2	1·4
Spinal cord	31·8	2·9	10·9***	1·7	2·3	0·9	3·9	0·7

Mean values were significantly different from those of Co-sufficient sheep (Student's t test): ** $P < 0.01$, *** $P < 0.001$.

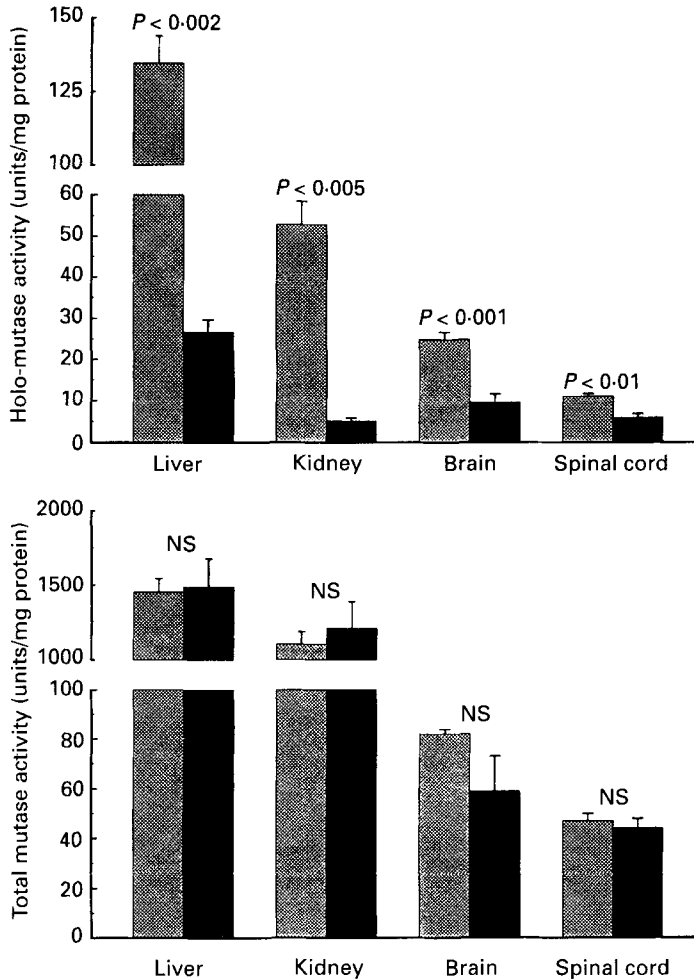† For details of animals and procedures, see pp. 68–69.

Fig. 3. Methylmalonyl-coenzyme A mutase (*EC* 5.4.99.2) activity in sheep tissues. Sheep were fed on either a cobalt-sufficient diet (*n* 4; 1000 ng Co/g; (▨)) or a Co-deficient diet (*n* 5; 4·5 ng Co/g; (■)) for 28 weeks. Holo-enzyme activity was measured radio-enzymically in tissue homogenates and total enzyme activity in the same homogenates, made with respect to 5′-deoxyadenosyl cobalamin under dim red lighting. Values are means with their standard errors represented as vertical bars. The statistical significance of differences between means were assessed using Student's *t* test. For details of animals and procedures, see pp. 68–69.

CoA mutase was significantly lower in all tissues of the animals fed on the Co-deficient diet than in the Co-sufficient controls (Fig. 3).

Odd-numbered branched- and straight-chain fatty acids in tissues
(20 and 28 week depletion)

The only significant difference in odd-numbered branched- and straight-chain fatty acids observed in the animals depleted of dietary Co for 20 weeks was an increase in the concentration of C_{15} branched-chain fatty acids in the kidney (Table 2). However, in the animals depleted of dietary Co for 28 weeks there were significant increases in C_{15} and C_{17} branched-chain fatty acids in all the tissues examined, by comparison with the controls. The

Table 2. *Odd-numbered straight-chain and branched-chain fatty acid concentrations in the tissues of sheep deprived of dietary cobalt for 20 or 28 weeks‡*

Tissue	Fatty acid		20 week depletion (μg fatty acid/g tissue)				28 week depletion (μg fatty acid/g tissue)			
			+Co (n 4)		−Co (n 4)		+Co (n 4)		−Co (n 5)	
			Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Liver	C15:	Straight	147·8	11·9	196·8	38·6	134·8	10·6	147·0	18·9
		Branched	52·1	41·8	41·8	7·8	35·5	8·8	78·5†	10·1
	C17:	Straight	569·7	117·8	879·2	235·5	498·8	40·3	468·0	69·5
		Branched	335·0	21·7	416·3	57·8	223·3	13·3	422·0***	39·1
Kidney	C15:	Straight	42·4	1·0	72·1	11·6	62·9	5·4	57·2	82·0
		Branched	5·0	0·5	9·0**	0·6	6·4	0·7	15·5*	3·3
	C17:	Straight	166·3	13·9	250·5	42·9	155·0	38·6	241·8	32·5
		Branched	62·8	4·9	97·2	12·1	74·8	10·7	179·0†	26·7
Brain	C15:	Straight	33·1	1·2	44·3	7·5	47·0	2·8	72·2***	3·1
		Branched	5·8	0·4	10·2	2·7	3·5	0·3	8·9**	1·0
	C17:	Straight	98·0	5·8	128·2	8·6	142·8	10·0	166·5	8·9
		Branched	27·2	1·6	37·5	3·7	40·7	2·3	85·7**	9·7

Mean values were significantly different from those for Co-sufficient sheep (Student's t test): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, † $P < 0.02$.

‡ For details of animals and procedures, see pp. 68–69.

only change in the concentration of straight-chain fatty acids occurred in the brain of animals fed on the Co-deficient diets for 28 weeks.

DISCUSSION

The use of plasma concentrations of MMA as a diagnostic indicator of functional vitamin B_{12} deficiency has increased markedly in both human and veterinary medicine over the last 10 years. An earlier study from this laboratory (Kennedy *et al.* 1990 *a*) showed increased MMA concentrations in plasma and tissues and decreased MM-CoA mutase activity in tissues taken from sheep fed on a Co-deficient diet for 20 weeks. However, the present study suggests that MMA has limitations to its value as a diagnostic indicator. As Co deficiency becomes progressively more severe, plasma MMA concentrations fall to levels indistinguishable from those in control lambs (Fig. 1). This finding was reflected in tissues, where MMA concentrations in Co-deficient animals were not significantly different from those in Co-sufficient controls (Table 1). One possible explanation for this could be that the animals were not depleted of vitamin B_{12}. This was shown not to be the case, however, since there were low concentrations of vitamin B_{12} in plasma and tissue (Fig. 1 and Table 1) and reduced activity of MM-CoA mutase (Fig. 3) in all tissues examined. Similarly, the activity of methionine synthase (*EC* 2.1.1.13) was also significantly reduced in the tissues of these Co-deficient lambs (Kennedy *et al.* 1992 *a*). We are not aware of any studies which have measured plasma MMA concentrations in severe Co deficiency. Ulvund (1990) showed a decrease in plasma MMA in late summer in field cases of Co deficiency. However, in those studies the plasma concentration of vitamin B_{12} rose at the same time as the decrease in MMA concentrations. This suggested that those animals were receiving an increased amount of dietary Co at that time. The reduction in the plasma concentration of MMA and

the normal concentration of MMA found in tissues at slaughter in the present study may, however, have been a consequence of the dramatic reduction in appetite experienced by the Co-deficient lambs towards the end of the experiment. This finding suggests that determination of plasma MMA concentrations may only be of value as a diagnostic indicator in moderate Co deficiency.

The lambs fed on the Co-deficient diet developed the appetite reduction and decreased live-weight gain characteristic of ovine Co deficiency. The changes in VFI closely paralleled the changes in live-weight gain.

Feeding diets low in Co to lambs for 20 weeks decreased tissue activities of MM-CoA mutase to 25% of control values in liver and to 12% of control values in kidney (Kennedy et al. 1990a). No changes in MM-CoA mutase activity were seen in either brain or spinal cord in that study. In the present study the decreases in enzyme activity in liver and kidney (to 20 and 10% of control values respectively) were similar to those reported earlier. However, in contrast to our earlier study, we have found that a 28-week period of Co depletion significantly decreased MM-CoA mutase activity in brain and spinal cord to levels which were 38 and 80% respectively of those observed in lambs fed on the Co-sufficient diet (Fig. 3). However, despite the increased severity of the Co deficiency induced, the tissue concentrations of MMA in the Co-deficient lambs were not significantly different from those observed in Co-sufficient controls (Table 1). This contrasts dramatically with our earlier study (Kennedy et al. 1990a) and may have been a consequence of the appetite reduction which occurred at a late stage in the development of Co deficiency (Fig. 2b) in the present study.

The concentrations of odd-numbered straight- and branched-chain fatty acids were similar in the tissues of lambs fed on the Co-sufficient diets for 20 and 28 weeks (Table 2). However, the only significant change in the tissue concentrations of odd-numbered straight- and branched-chain fatty acids in lambs fed on the Co-deficient diet for 20 weeks, in comparison to their Co-sufficient controls, was an increase in the concentration of C_{15} branched-chain fatty acids in kidney (Table 2). This change occurred because of the decreased activity of MM-CoA mutase and increased concentrations of MMA in the liver and kidney of these animals (Kennedy et al. 1990a).

In lambs fed on the Co-deficient diet for 28 weeks increased concentrations of C_{15} and C_{17} branched-chain fatty acids were observed in liver, kidney and brain (Table 2), by comparison with controls. It is significant that these increases occurred against a background of low tissue MMA concentrations. It is possible, thus, that the decrease in tissue MMA concentrations might have been due to the reduction in appetite as suggested for plasma MMA. However, it might also have been due to an increased incorporation of MM-CoA into the branched-chain fatty acids, since the accumulation of these fatty acids does appear to have occurred very late in the course of this disease (Table 2). This suggestion is also consistent with the finding by Frenkel et al. (1973) that fatty acid synthesis was increased in the tissues of vitamin B_{12}-deficient rats.

The only significant change in straight-chain fatty acids was observed in brain in which there was an increased concentration of pentadecanoic acid (C_{15}) (Table 2). These findings show that misincorporation of MMA into odd-numbered branched-chain fatty acids occurs at a greater rate than misincorporation of propionate into odd-numbered straight-chain fatty acids. This finding contrasts with the results obtained by Duncan et al. (1981) who showed increased concentrations of odd-numbered straight-chain fatty acids in the tissues of lambs born to Co-deficient ewes. The reasons for this difference are not clear, but may result from differences in the permeability of the placenta to propionyl-CoA and methylmalonyl-CoA. Smith et al. (1969) showed that the CoA concentration of Co-deficient sheep was 2·6 times higher than that in Co-sufficient controls. They suggested

that MM-CoA accumulation accounted for a small proportion of the increased CoA pool. However, they did not identify the compounds which were responsible for this accumulation.

Vitamin B_{12} deficiency is known to increase fatty acid synthesis in rats (Frenkel et al. 1973). This finding, together with the knowledge that propionyl-CoA and MM-CoA could be misincorporated into fatty acids, led to an association between deranged fatty acid metabolism and the development of neurological abnormalities in man (Frenkel, 1973; Kishimoto et al. 1973; Ramsey et al. 1977). However, the increases in the tissue concentrations of branched-chain fatty acids reported in the present study were not associated with the development of any clinical signs indicative of neurological damage, nor was there histopathological evidence of neurological lesions, in these animals (S. Kennedy, unpublished). This finding may indicate that accumulation of these abnormal fatty acids is unrelated to the development of neurological lesions and that a perturbation of methylation, as a consequence of inhibition of methionine synthase, is central to the development of these lesions (Weir et al. 1988). Evidence to support this hypothesis was obtained previously when we reported that there was no change in the activity of methionine synthase in the neural tissues of the sheep used in the present study (Kennedy et al. 1990a, 1992a). Sheep are known to metabolize approximately 1 mol propionyl-CoA to succinyl-CoA daily, mainly in the liver. Given the significant reduction in MM-CoA mutase activity observed in the tissues of Co-deficient sheep in both the present study (Fig. 3) and our earlier study (Kennedy et al. 1990a), large increases in the tissue concentrations of odd-numbered straight- and branched-chain fatty acids might have been expected. However, we recently discovered that rumen production of propionate is greatly reduced in ovine Co deficiency, and that high concentrations of succinate accumulate in the rumen instead, within days of switching sheep to a Co-deficient diet (Kennedy et al. 1991b). The absorption of succinate from the rumen and its potential for subsequent utilization in the liver suggest that the ability of the Co-deficient sheep to synthesize large amounts of branched-chain fatty acids might be considerably lower than would otherwise be predicted.

In conclusion, we have shown that long-term Co deficiency in sheep causes a reduction in MM-CoA mutase activity in all tissues studied. The consequent accumulation of branched-chain fatty acids may have been caused by misincorporation of MM-CoA into fatty acids in place of malonyl-CoA. Although these are abnormal fatty acids, their accumulation in these animals was not associated with the development of any of the neurological or histopathological signs which have previously been attributed to them. It is possible, however, that the reduced rumen synthesis of propionate which accompanies ovine Co deficiency may have helped to mitigate possible adverse effects of abnormal accumulation of branched-chain fatty acids in tissues.

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